BIOPROCESSING CONDITIONS FOR IMPROVING MATERIAL PROPERTIES OF TISSUE ENGINEERED CARTILAGE

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BIOPROCESSING CONDITIONS FOR IMPROVING MATERIAL PROPERTIES OF TISSUE ENGINEERED CARTILAGE

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TO

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CS	Chondroitin Sulfate
DAB	Diamethylaminobenzaldehyde
dI	Deionized
DMEM	Dulbeco's modified Eagle medium
DMMB	Dimethyl methylene blue
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FBS	Fetal bovine serum
GAG	Glycosaminoglycan
H & E	Hematoxylin & Eosin
NO	Nitric Oxide
PBS	Phosphate buffered saline
TGF	Transforming growth factor

SUMMARY

Tissue engineering is considered as a potential therapeutic option for the regeneration of damaged cartilage tissue. Bioreactors are devices engineered for the application of specific stimuli on tissue constructs to promote matrix deposition. Currently, tissue engineered cartilage has lower matrix content and material properties compared to native tissue. Establishing relationships between culture conditions and tissue composition is important for tissue engineering. This study identifies certain specific bioreactor conditions that enhance construct extracellular matrix production. Experiments are carried out based on the hypothesis that bioreactor conditions specifically, fluid induced shear stress and oxygen tension affect construct extracellular matrix deposition. The results suggest that a combination of biochemical and mechanical stimuli as opposed to either stimulus alone may be required to enhance construct extracellular matrix deposition. In order to increase nutrient transport to the construct and use multiple fluid flow regimes, a perfusion shear flow bioreactor was designed and a prototype built. The constructs cultured in this bioreactor showed robust cell proliferation and extracellular matrix deposition over short term (14 day) and long term (35 day) cultures.

In summary, this work focuses on specific bioreactor culture conditions that can be used to increase extracellular matrix deposition and enhancement of bioreactor design.

INTRODUCTION

One in seven Americans suffers from a musculoskeletal impairment and as many as $215 billion are spent annually for the treatment of these conditions. Among these conditions, arthritis is the most commonly reported problem. As many as 17 million people were reported to be suffering from knee problems in the year 2000 alone (2004; Praemer et al., 1999). The most common type of impairment results from osteoarthritis, where the cartilage covering the ends of the joint gradually wears away, exposing the bone ends. This makes joint motion difficult and painful. Self-repair of cartilage is limited and the repair tissue that is formed is a combination of hyaline and fibro-cartilage. This repair tissue does not perform as well as hyaline cartilage and degrades over time. Furthermore, the repair tissue usually lacks the mechanical properties and ultrastructure to ensure long-term stability (Lonner, 2004). Current therapies for osteoarthritis include subchondral shaving, autologous grafts, and mesenchymal cell regeneration therapy. Some of these therapies work in the short term for small lesions. However, no long term treatment strategy is available for the repair of cartilage in full thickness lesions damaged due to osteoarthritis and related joint disorders.

Tissue engineering is considered as a potential therapeutic option for the regeneration of damaged tissue. Tissue engineering can perhaps be best described as the use of a combination of cells, engineering materials, and

suitable biochemical and mechanical factors to improve or replace biological tissue to effect the advancement of medicine. A tissue engineered cartilage template or construct could potentially repair full thickness defects in a diarthrodial joint. However, as in the case of most tissue engineered substitutes, the requirements of culture conditions and the definition of suitable implants are still open to debate. In the case of cartilage tissue engineering, this challenge is only enhanced by the complex architecture and material properties of native cartilage.

Native cartilage is a highly organized tissue with a well-defined zonal cell morphology and matrix fibril organization. The tissue has low cell volume and an abundance of extracellular matrix. Cartilage exhibits a zone specific organization of matrix fibrils that is responsible for the material properties of the tissue. The functional ability of cartilage depends on the organization and quantity of extracellular matrix and its material properties. Thus, long-term successful repair or regeneration of articular cartilage in synovial or weight bearing joints, such as the knee or hip, is a formidable task not currently achievable through surgical and non-surgical treatments. One approach to tissue engineering of cartilage is to seed chondrocytes or mesenchymal progenitor cells onto biodegradable polymer scaffolds in a culture environment that promotes the development of tissue suitable for implantation.

Bioreactors are devices engineered for the application of specific stimuli (mechanical and biochemical) on tissue constructs to promote matrix deposition. Currently, engineered tissue has lower matrix content and material properties

compared to native tissue. Very few studies have assessed the matrix organization within engineered tissue constructs. The bioreactor environment provides the necessary biochemical and mechanical conditions (e.g. shear stress, hydrodynamic loading, perfusion) to stimulate chondrocytes to synthesize and deposit extracellular matrix. The application of complex, multi-factorial bioreactor environments could, in theory, provide structural materials suitable for implantation in vivo.

Many bioprocessing stimuli promote cartilage extracellular matrix deposition. In vivo, cartilage experiences shear stress and compression as primary forces. In vitro, shear stress affects cartilage matrix deposition. However, the degree of stimulation that can be achieved using shear stress is not known. Establishing relationships between culture conditions and tissue architecture will lead to the development of tissue engineered cartilage with improved function for in vivo performance. This research tests the hypothesis that bioreactor operating conditions, specifically fluid induced shear stress and oxygen tension, affect the extracellular matrix deposition in tissue engineered cartilage constructs. This study identifies certain bioreactor conditions that increase the deposition of matrix components in the engineered constructs by means of bioprocessing conditions – both biochemical and physical, and correlating the extracellular matrix content of the tissue construct to its culture environment and mechanical strength. This research addresses some fundamental issues that promote development of functional cartilage production in vitro.

Objective 1: Develop quantitative relationships between construct hydrodynamic loading, oxygen tension and material properties in a concentric cylinder bioreactor

Matrix synthesis and deposition depends upon chondrocyte loading conditions. In order to identify the effect of the hydrodynamic loading regimen and nutrient environment in the bioreactor, experiments were carried out under defined bioreactor conditions. The hydrodynamic loading was varied over the culture period and the kinetics of matrix production quantified. Bioreactor experiments combining low oxygen tension and hydrodynamic loading were carried out to determine the effect of simultaneous stimuli on extracellular matrix synthesis. The material properties of the constructs were measured to examine their load-bearing capability.

Objective 2: Design and development of a perfusion bioreactor system for engineered cartilage constructs.

Perfusion can be used to increase chondrocyte cell proliferation and matrix synthesis within the construct. The concentric cylinder bioreactor configuration was modified to incorporate perfusion through the constructs. The new bioreactor design exposes cells within the construct to fluid flow, diminishes nutrient gradients, and provides hydrodynamic loading to stimulate chondrocytes to produce more extracellular matrix. The perfusion concentric cylinder bioreactor attempts to provide a uniform inter-construct environment with the ability to introduce intra-construct heterogeneity.

This work identifies specific bioreactor operating conditions that increase both construct extracellular matrix content and desirable material properties. In

addition, it has led to the development of a new bioreactor design that allows for a uniform inter-construct and non-uniform intra-construct hydrodynamic environment. Thus, this work identifies specific culture conditions that are necessary for the development of cartilage constructs with enhanced material properties for potential in vivo application.

BACKGROUND

Biochemistry of Cartilage

Articular cartilage is a thin layer of soft connective tissue that overlies the articulating bony ends of diarthrodial joints. It provides for the distribution of mechanical loading, resistance to compressive forces, and cushioning of the subchondral bone during joint movement (Kuettner et al., 1991). The primary role of articular cartilage is to transmit mechanical stresses across the articulating joint. The load bearing capability of cartilage is a result of the abundance of extracellular matrix (ECM) that the tissue contains. The ECM in cartilage can be viewed as a complex biocomposite material that exhibits compressive stiffness, toughness, strength, resilience, and shock absorption.

Chondrocytes are the only cell type present in cartilage. These cells are embedded in a complex extracellular matrix consisting of proteoglycans and collagen, with little to no cell-cell contact. The proteoglycans make up to 5-10% of tissue wet weight, the majority of them being glycosaminoglycan (GAG) molecules. A large fraction of cartilage is type II collagen, up to 10-30% of tissue wet weight. Healthy cartilage is a highly hydrated tissue and has between 65-80% water (Kuettner et al., 1991). The extracellular matrix consists of many complex carbohydrates and proteins with a high electronegative charge that allows for binding large amounts of water.

Chondrocytes synthesize, secrete and organize the extracellular matrix during different stages of development (Muir, 1995). Chondrocytes are protected

from the potentially damaging forces of normal mechanical function by their surrounding ECM. In vivo, the chondrocytes are constantly subject to changes in their hydrostatic environment that modulate their metabolic activities (Manicour et al., 1999). Since cartilage is avascular, chondrocyte nutrition depends mainly on the diffusion of nutrients through the synovial fluid into the matrix. The oxygen tension in cartilage can be as low as 1-3% and the cells are well adapted to these conditions (Brighton and Heppenstall, 1971). Anaerobic glycolysis provides a large part of chondrocyte energy needs because of the hypoxic environment.

The most abundant component of cartilage is collagen, mainly type II with collagen IX and XI being present in small quantities. Collagen type II forms up to 50% of the dry weight of the tissue (Eikenberry E.F and Bruckner, 1999). Collagen type II has a glycine rich amino acid sequence of glycine-X-Y where X and Y are hydroxyproline and proline arbitrarily. The collagen network in cartilage provides tensional stability and mechanical strength. This is achieved by collagen fibers linking to glycoproteins and proteoglycans. These linking molecules also regulate fibrillogenesis by preventing accumulation of new collagen molecules to pre-existing fibrils. By forming a fibrous network and interacting with proteoglycans and water, collagen serves to maintain the shape and form of the tissue under large forces. Collagen types IX and XI also found in cartilage are fibril-forming collagens. Type IX collagen has extensive cross-linking capabilities while Type XI collagen forms a core on which type II collagen molecules are deposited (Manicour et al., 1999; Sandell and Aigner, 2001).

Non-collagenous proteins contribute to the regulation of tissue assembly and mechanical properties. Aggrecan provides fixed charge groups, creating an osmotic environment in cartilage, immobilizing and restricting interstitial water flow. The interaction between proteoglycans and collagen fibers provides cartilage with resistance to compressive and shear forces (Mow and Wang, 1999; Muir, 1995; Stading and Langer, 1999). Additional structure is provided by cartilage oligomeric matrix protein (COMP) (Di Cesare et al., 2002; Stading and Langer, 1999). COMP is abundant near chondrocytes and may stabilize the collagen network, promote collagen fibril assembly, and regulate cell-matrix interactions. The proteoglycan component in the extracellular matrix consists of a highly concentrated gel (up to 100 mg/ml) of proteoglycan immobilized in a dense network of collagen fibrils. Chondroitin sulfate and keratan sulfate are the most abundant cartilage GAGs. Keratan sulfate can account for between 5- 20% of total GAG in cartilage. Hyaluronic acid is the only non-sulfated proteoglycan. Although it is present in small quantities in human cartilage (< 6% of total weight), it performs a very important biochemical function in the formation of proteoglycan aggregates. Under normal physiological conditions, most proteoglycans exist as huge multi-molecular aggregates bound to hyaluronic acid and a link glycoprotein (Hardingham et al., 1983; Plaas et al., 1983).

Cartilage Ultrastructure

Cartilage is divided into three zones: superficial, transition and deep zone. The cell morphology and collagen fiber orientation differs in each of these zones. In the superficial zone, the collagen fibrils are oriented tangential to the

articular surface and are very closely packed (Figure 1). The fibers in this region are typically 320 ± 50 Å with a periodicity of 100 Å. The pericellular matrix has finer fibers about 120 Å in diameter. Collagen fibers in the transition zone have a periodicity of 640 Å and their diameter varies from 300 to 600 Å. The diameter of fibers in the deep zone varies between 400 and 800 Å and the periodicity is 640 Å. In the middle and deep zones, collagen fibrils have a randomly oriented appearance with the fibers being very widely spaced (Zhu et al., 1993).

Chondrocytes in the superficial zone are typically oval in shape, with the long axis of the cells oriented along the articular surface (Figure 2). Chondrocytes in the transition zone have a more rounded profile while the cells in the deep zone are arranged preferentially in a vertical direction relative to the articular surface (Hunziker et al., 2002). Differences are found in the chondrocytes in terms of cell volume and surface area in the superficial zone and radial zone (Hunziker et al., 2002; Wong et al., 1996). The cells in the radial zone have a much higher (~ 10 times more) biosynthetic activity as compared to cells in the superficial zone.

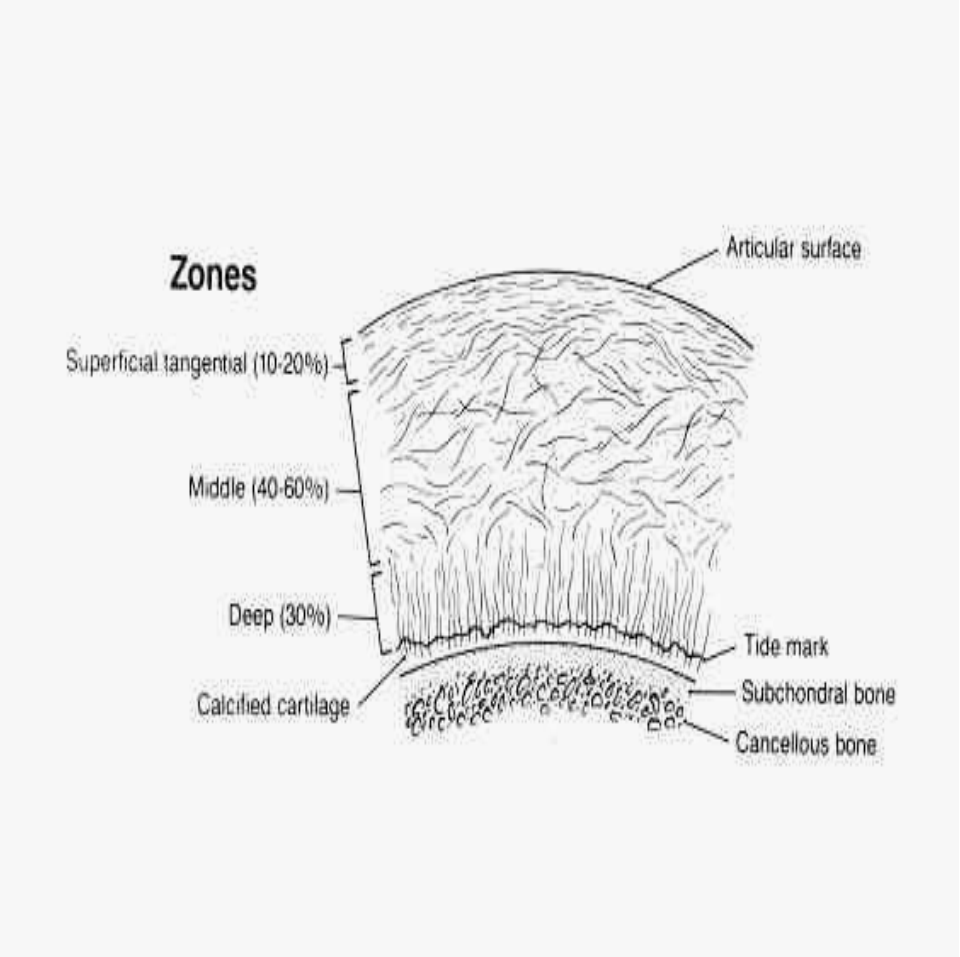


Figure 1: Collagen fibril organization in native articular cartilage. The collagen fibrils are organized parallel to the articular surface in the surface zone, randomly in the middle zone and perpendicular to the calcified cartilage in the deep zone. (Ratcliffe and Mow, 1996)

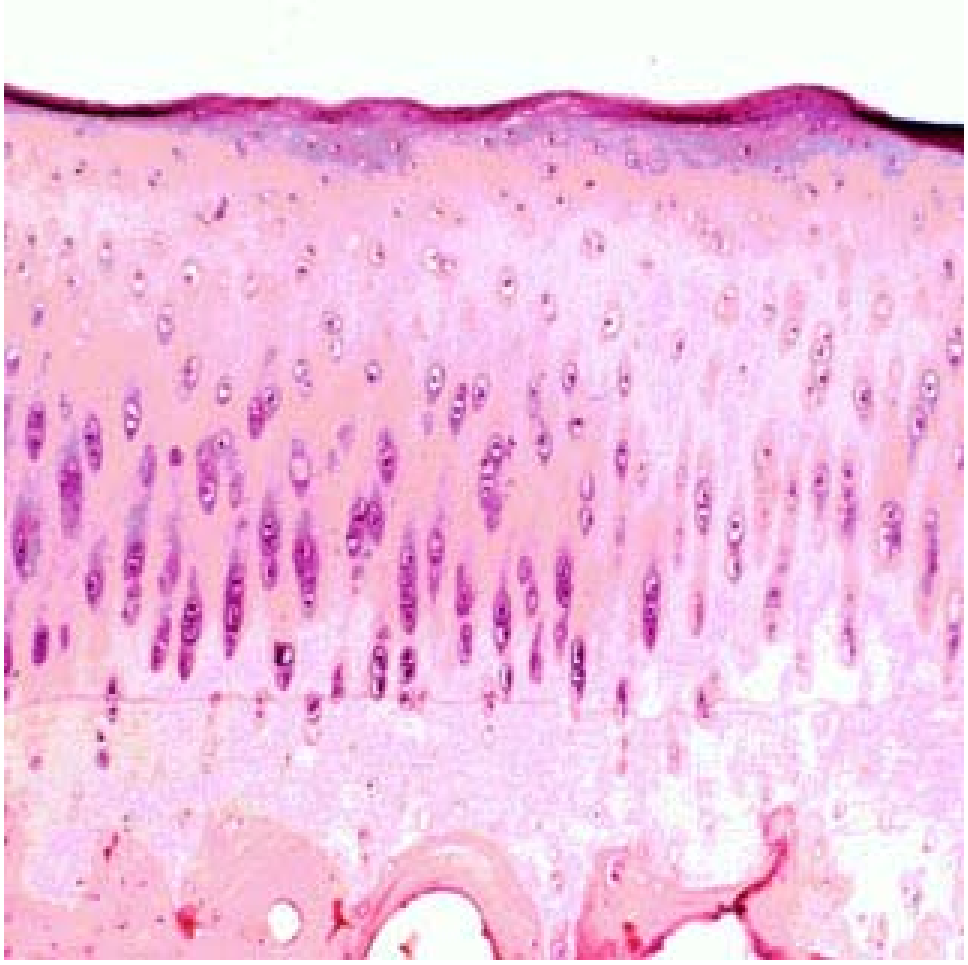


Figure 2: Histological image of chondrocyte organization through the full thickness of articular cartilage. The chondrocytes are arranged parallel to the articulating surface and are arranged as perpendicular cascades near the calcified cartilage. (Image courtesy: http://dahweb.engr.ucdavis.edu/dahweb/126site/tidemrk.htm)

Heterogeneity within the native tissue has been observed in the collagen fibril distribution and molecular weights of proteoglycan aggregates (Lonner, 2004; Roy and Meachim, 1968; Mow et al., 1984). Changes in cell shape in the zones are postulated to be an important mechanotransducer by which the biosynthetic activity of chondrocytes is regulated (Hunziker et al., 2002; Wong et al., 1996).

Techniques using confocal microscopic stereology have been developed to give insight into the structural parameters of the tissue (Wong et al., 1996). These findings suggest that the chondrocytes harvested from different cell layers have different matrix synthesis rates.

Cartilage Material Properties

Cartilage loading can reach 20 MPa during normal activity. Application of external load causes deformation, increases hydrostatic pressure and interstitial fluid flow (Davisson et al., 2002a) throughout the extracellular matrix. The load is transmitted through the chondrocyte pericellular matrix and detected by cells through a variety of signaling pathways (Urban, 2000). Native bovine cartilage has an aggregate modulus in the range of 0.47-0.89 MPa and dynamic shear modulus in the range of 0.2-2.5 MPa (Mow et al., 1984; Ratcliffe and Mow, 1996). The actual values depend on the location of the tissue specimen. In addition to tissue location, the material properties also depend on tissue composition and ultrastructure. The layered morphology of the collagen network and the orientation of the fibrils dictate the mechanical properties and the load bearing abilities of the tissue (Mow et al., 1984).

Zone specific analysis of adult native cartilage is important for understanding the structure-function relationship of native cartilage. The material properties of cartilage are known to vary with tissue location and orientation. Permeability of native cartilage decreases with tissue depth; the decrease varying with distance from the articular surface. Variable permeability is thought to provide a valuable mechanism for load distribution between the solid and fluid phases of cartilage. Other studies propose that the anisotropy in the permeability of cartilage could be due to the GAG network geometry. It has been suggested that the GAG network is not randomly oriented but there might be preferred GAG orientations and directionally dependent reduction of inter-GAG spacing (Quinn et al., 2001).

The biphasic model for cartilage shows that the cartilage exhibits significant anisotropy in its mechanical properties mainly due to the changes in the collagen fibril orientation in the different zones of the tissue (Poole et al., 2001; Lonner, 2004). The layered morphology of the collagen network (heterogeneity) and the orientation of the fibrils seem to dictate the mechanical properties and the load bearing abilities of the tissue (Lonner, 2004; Caplan and Goldberg, 1999). The superficial zone provides the highest tensile properties found in cartilage; therefore it can accommodate forces due to compression, shear and tensile forces during articulation. The middle and deep zone provide intermediate properties between those of the superficial zone and the calcified bone.

Osteoarthritis

Osteoarthritis is one of the most common types of arthritis characterized by the degeneration of cartilage. Degradation of cartilage in osteoarthritis is characterized by two phases: phase one, where the chondrocytes attempt to repair the damaged ECM with little success and phase two, where the activity of the enzymes produced by chondrocytes digests the ECM and matrix synthesis is inhibited. In osteoathritic cartilage, chondrocytes continue to produce the matrix components; however they are unable to keep up with the rate of degradative catabolic activity and tissue degradation results.

Studies indicate that enzymes such as collagenases and aggrecanases cause the degeneration of cartilage in osteoarthritis. Collagenases (also known as matrix metalloproteinases) cause the degradation of the triple helix of collagen allowing for further degradation by aggrecanases. Collagenases make an initial cleavage in a weak point in the collagen fibril and then the aggrecanases cleave the core protein (Mort and Billington, 2001),(Roughley, 2001). Aggrecanases, in tandem with other enzymes cause the degradation of aggrecan. These changes in the matrix components of cartilage cause it to weaken and therefore not be able to perform its biomechanical function.

Articular cartilage exhibits very low capacity for self-repair because of its avascularity. The repair tissue is a mixture of hyaline and fibro-cartilage and consists mainly of type I collagen as opposed to type II collagen. Additionally, the repair tissue possesses inferior mechanical properties when compared to healthy tissue. The change in composition and inferior biomechanical properties

of spontaneously formed repair cartilage contribute to the poor functionality of the repaired tissue (Roughley, 2001; Altman et al., 1992).

Diseased cartilage shows a distinctly different ultrastructure compared to healthy tissue. Osteoarthritic cartilage shows vertical orientation of the collagen fibers throughout the thickness of the cartilage, right up to the superficial zone. The articulating surface is less smooth compared to healthy adult tissue. The water content of the diseased tissue is higher, increasing the permeability and reducing the compressive modulus of cartilage (Roy and Meachim, 1968).

Current treatment strategies available for cartilage regeneration include osteochondral grafting, autologous chondrocyte implantation, and mesenchymal stem cell regeneration. Transplantation of autologous mesenchymal progenitor cells leads to filling of the defect with tissue that is less stiff and more compliant than the native tissue. This suggests that the regenerated tissue may have inferior mechanical properties compared to healthy tissue. Fresh osteochondral allografts have been transplanted in defect sites to study their potential for regeneration of functional cartilage (Lonner, 2004). The success of this therapy is limited by the availability of graft tissue, viability of chondrocytes in the graft post-implantation, and careful patient and donor selection.

Some of the surgical treatment methods are shaving of fibrillated articular cartilage and abrasion of the subchondral bone to allow for penetration of the subchondral plate and induction of chondrogenesis (Farnworth, 2000). Osteochondral shaving and subchondral abrasion of the articular cartilage are potential treatment therapies for large, full thickness defects (Kim et al., 1991).

However, these methods do not fully aid regeneration of new tissue or completely restore tissue function. Electron micrographs of the repair sites show that no new superficial zone is formed and reconstruction of a smooth congruent surface is not possible by these methods. The results from these studies are unpredictable, and are believed to be temporary. Lack of long-term success with surgical therapies has motivated the development of tissue engineering of cartilage constructs in vitro for implantation.

Tissue Engineering

Tissue engineering of cartilage in vitro is a promising option for the production of implantable cartilage templates. This area of biotechnology has been defined at the first scientific meeting devoted to tissue engineering in 1988 at Lake Tahoe, California as "the application of the principles and methods of engineering and life sciences toward the fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain or improve function". Some of the major challenges in tissue engineering include cell-sourcing, biomaterials for scaffold development, culture environment including bioreactor design and meeting patient demand.

For in vitro studies chondrocytes have been harvested from cows (Martin et al., 2000), sheep (Neves et al., 2002), rabbits (Dunkelman et al., 1995) etc. Mesenchymal progenitor cells have been used to study their potential for differentiation into chondrocytes in vitro and production of cartilage constructs (Martin et al., 1998; Barry et al., 2001). More recently, human mesenchymal cells

have been used for the production of cartilaginous tissue in vitro (Neves et al., 2002). Biodegradable scaffolds seeded with chondrocytes and cultured in vitro allow the chondrocytes to proliferate and synthesize matrix. These cell-seeded scaffolds allow for the development of tissue that is biochemically and morphologically similar to native tissue. The seeding of chondrocytes on synthetic biodegradable matrices provides a large surface area for cell attachment and proliferation (Vacanti et al., 1991).

Matrix scaffolds allow the chondrocytes to retain their differentiated phenotype and provide a three dimensional framework for tissue growth. Ideally, the scaffold should possess a three dimensional structure that allows for high cell seeding densities, minimize diffusional constraints for nutrient transfer and degrade at a rate comparable to the rate of ECM synthesis (Freed et al., 1999). Polyglycolic acid (PGA) meshes support chondrogenesis from initial high cell densities (Freed et al., 1998).

Biodegradable poly L-Lactic acid (PLLA), PGA and poly lactide-galactide (PLGA) copolymer meshes have been used in different studies for the in vitro growth of cartilage (Freed et al., 1993a; Freed et al., 1994c). Polyphospazenes, polyanhydrides, polypropylene fumarate are some other biodegradable polymers that are being investigated for in vitro culture of chondrocytes (Behravesh et al., 1999). Studies on copolymers of PLLA and PGA have shown that the construct properties depend mainly on the composition of the scaffold. The ratio of PLLA and PGA in the scaffold affects the chondrocyte adherence, proliferation and mechanical properties (Moran et al., 2003). Increasing the amount of PLLA in

the scaffolds increases the compressive modulus and characteristic degradation time of the scaffolds. However, the cell seeding efficiency and proliferation decreases with increase in the amount of PLLA. The choice of the biomaterial is thus very important for the development of cartilage templates.

Chondrocytes tend to retain their phenotype in vitro when cultured under conditions that resemble their natural environment. The mechanical and biochemical signals that affect cartilage development in vivo are very likely to promote tissue growth in vitro. Ideally the engineered tissue construct should be similar to native cartilage with respect to composition, matrix organization and mechanical properties. A culture environment that incorporates the different factors that promote matrix component synthesis is essential for the production of functional cartilage constructs.

Bioreactors in Cartilage Tissue Engineering

The basic hypothesis for tissue engineering cartilage is that manipulating the physical and chemical environment of the cell seeded scaffold composite will modulate the structure and function of engineered cartilage (Freed et al., 1999). Bioreactors are useful tools to incorporate the appropriate physical and chemical environment for tissue engineered cartilage constructs. Some of the main reasons why bioreactor culture enhances cartilage development in vitro are (a) enhanced nutrient and oxygen transport to the constructs is possible because of the mixing patterns, (b) spatially uniform cell seeding and nutrient delivery is possible because of bioreactor design, and (c) the hydrodynamic forces stimulate the chondrocytes toward increased matrix synthesis as compared to static

culture (Freed et al., 1993b; Ratcliffe and Niklason, 2002; Darling and Athanasiou, 2003) . Some of the commonly used bioreactor configurations used are shown in Figures 3 and 4.

Spinner flasks

Spinner flasks are simple bioreactors that provide a turbulent fluid flow environment to alleviate nutrient and oxygen diffusion limitations. Spinner flasks were one of the first bioreactor configurations used to grow tissue engineered constructs with cartilaginous composition and appearance. These bioreactors are characterized by a high seeding efficiency and simple geometry. Typically, scaffolds seeded with chondrocytes are attached to needles suspended from a stopper in the flask. Media is added to cover the scaffolds and mixing is maintained with a magnetic stir bar in the bottom of the flask. In order to alleviate diffusion limitations, the culture medium is continually stirred. Stirring exposes the tissue to fresh nutrients and also shear forces. Spinner flasks have their inherent drawbacks in terms of difficulty of scale-up and also individual handling. Also, the fluid flow is turbulent and not uniform throughout the bioreactor. It is difficult to define the flow field around the construct and the constructs in the spinner flasks experience non-uniform shear gradients, mass transfer and nutrient gradients (Ratcliffe and Niklason, 2002). The cartilage constructs cultured in spinner flasks have been characterized by the formation of a capsule on the outer edge of the construct (Ratcliffe and Niklason, 2002). These constructs have an outer capsule with increased cell density and an inner core with fewer cells. The constructs showed an increased release of GAG into the

media possibly due to the turbulent flow regime around the constructs. The wet weight percentage of GAG was lower in spinner flask cultivated constructs as compared to the constructs cultured in other bioreactors.

While spinner flasks have been successful as a bioreactor configuration for the development of cartilage constructs, certain limitations have been highlighted by these studies in bioreactor configuration. These drawbacks highlight the bioprocessing needs for the culture of cartilage constructs and have led to the design and development of bioreactors that eliminate turbulent flow regimes and nutrient gradients.

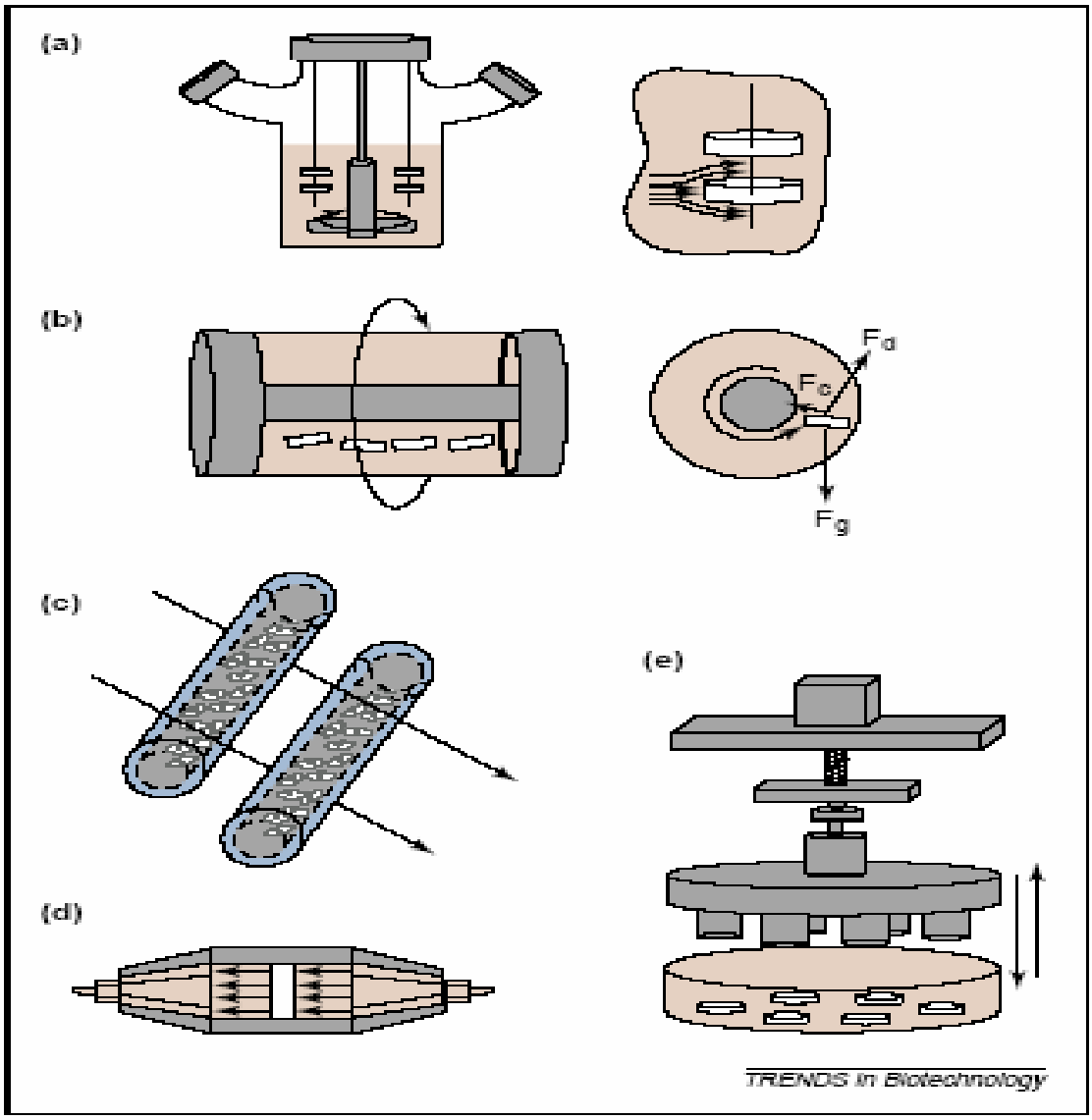


Figure 3: Different bioreactor configurations used in cartilage tissue engineering. (a) spinner flask, (b) rotating wall vessel bioreactors, (c) hollow fiber membrane bioreactors, (d) perfusion bioreactors, and (e) compression loading bioreactors. (Martin et al., 2004)

Rotating Wall Perfusion Vessel bioreactors (RWPV)

The rotating wall perfusion vessel bioreactor has been used successfully in the culture of tissue engineered cartilage construct. This bioreactor configuration has been used in long term studies for seven months to evaluate the material properties of the constructs. The RWPV is typically designed to provide a suitable environment for shear-sensitive mammalian cells. The RWPV, based on the viscous pump, has successfully cultured mammalian cells both on earth and in microgravity. The constructs cultured in these bioreactors in terrestrial environments showed a more uniform appearance as compared to the constructs cultured in the spinner flasks. The GAG and collagen wet weight fractions were relatively higher in these constructs as compared to constructs cultured in spinner flasks.

The rotating bioreactor developed at NASA's Johnson Space Center is configured as an annular space holding 110ml of culture medium and up to 12 constructs. The vessel is operated by solid body rotation in a horizontal plane. Vessel rotation can be adjusted so that the tissue constructs can be made to suspend in equilibrium between the acting forces or to rotate synchronously (Freed and Vunjak-Novakovic, 1997; Begley and Kleis, 2000; Begley and Kleis, 2002). The rotating wall bioreactor randomizes the effect of unit Earth gravity on freely suspended biological specimens and exposes them to dynamic fluctuations in fluid velocity, pressure and shear (Begley and Kleis, 2000; Begley and Kleis, 2002). This allows the constructs to be in a state of perpetual free fall. The integration studies with native tissue showed clearly visible empty spaces

between the engineered and native tissue (Obradovic et al., 2001). Also, long-term growth studies (seven months) showed that the mechanical properties of the tissue remained subnormal as compared to native tissue.

Perfusion Bioreactors

Perfusion culture is a way of establishing nutrient supply throughout the thickness of the construct, overcoming the diffusion limitations. In perfusion flow bioreactors, porous constructs are mounted in an impermeable housing, and fluid is forced through the construct. Several constructs can be perfused while having one media reservoir. A common design for perfusion bioreactors is usually to direct constant fluid flow through the developing tissue without stagnant or secondary flows.

Although the complete mechanism is not well understood, perfusion is known to modulate many changes in the in vitro growth of chondrocytes including increases in matrix synthesis. Perfusion increased the cellularity and matrix synthesis in the constructs as compared to static controls (Jin et al., 2001). A bioreactor design developed by Advanced Tissue Sciences Inc. forced influent media through the cultured constructs as media flows from the bottom to the top of the bioreactor vessel (Davisson et al., 2002b). Ample gas exchange and equal fluid flow distribution were demonstrated during the operational and sterility testing phases of the bioreactor. The axial flow through the sample induced in the bioreactor produced multiple focal areas of columnar cell orientation and assembly (Davisson et al., 2002b). The ultrastructure of constructs cultured under perfusion culture with intermittent fluid pressure showed the orientation of

collagen fibers around the chondrocytes with proteoglycan aggregates intertwined about collagen fibrils (Carver and Heath, 1999b; Carver and Heath, 1999a). The compressive modulus of the constructs cultured in the perfusion environment was higher compared to the constructs cultured in spinner flasks alone.

Concentric cylinder bioreactor (CCBR)

The main goal of this bioreactor is to provide a reduced shear stress environment for the efficient dynamic cell seeding in the constructs while causing minimal damage to cells (Saini, 2002; Saini and Wick, 2003). This bioreactor has a large surface area for construct growth and high productivity. Bioreactor design objectives include a low shear stress regime to minimize cell damage and allow for efficient dynamic seeding, a large growth area to increase construct production per unit reactor volume, and a simple geometry to allow for calculation of nutrient transport and hydrodynamic loading on the constructs (Figure 4).

The concentric cylinder bioreactor is based on a concentric cylinder viscometer design for cell culture (Saini, 2002; Saini and Wick, 2003). The bioreactor environment is homogeneous for tissue production because of the hydrodynamic and mass transport conditions provided by virtue of its design. The concentric cylinder bioreactor provides a homogenous, controllable flow environment across the construct. It has an easily scalable design and use of increased number of constructs does not greatly affect the flow field (Williams et al., 2002).

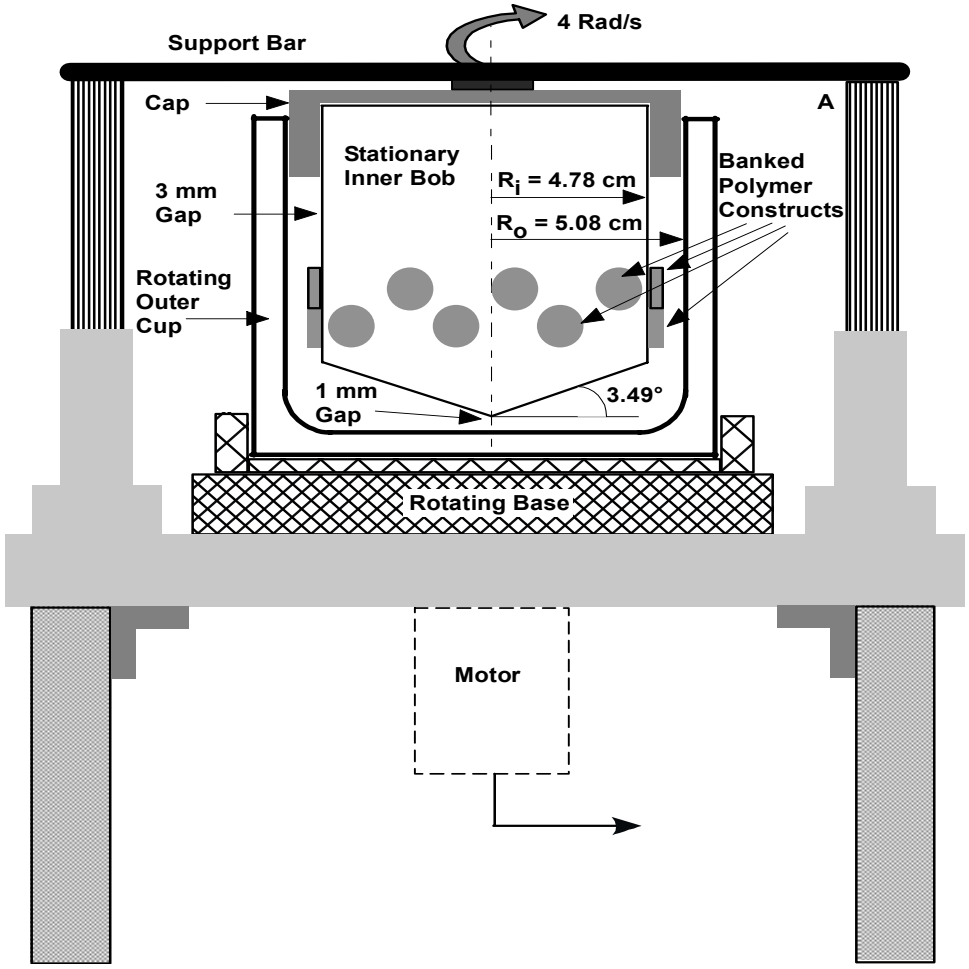


Figure 4: Concentric cylinder bioreactor. This bioreactor was designed in our lab by Dr. Sunil Saini and is based on the design of the concentric cylinder viscometer. The bioreactor consists of a stationary inner bob and a rotating outer cup (Saini, 2002; Saini and Wick, 2003).

Production of ECM in the concentric cylinder bioreactor varies with the outer cup rotation. Specifically, studies in a concentric cylinder bioreactor have shown that higher shear promotes glycosaminoglycan (GAG) synthesis early in culture (at 12 days), but the GAG synthesis drops at later time points with higher outer cup rotation rate. In contrast, lower shear prolongs the period of matrix GAG deposition up to 28 days (Saini and Wick, 2003). With low outer cup rotation rate, chondrocytes synthesized higher GAG at later time points of 2-4 weeks (Saini, 2002). These studies indicate that steady shear over the culture period alone may not be sufficient to increase extracellular matrix in cartilage constructs. This suggests that varying the outer cup rotation rate over the culture period in the bioreactor will lead to sustained production of matrix. Thus, chondrocytes exhibit a differential response to hydrodynamic loading, suggesting that chondrocyte matrix deposition kinetics can be regulated by modulating the shear stress in a controlled manner over the culture period.

Bioprocessing Conditions

Specific bioprocessing conditions have been shown to increase construct extracellular matrix deposition and induce a favorable environment for chondrogenesis in bioreactor culture. These conditions include low oxygen tension (hypoxia), fluid induced shear stress, mechanical loading, and growth factors. Some of these conditions that have a direct bearing on the current research on our work are reviewed here.

Low oxygen tension

Articular cartilage experiences low oxygen tension in vivo and the cells carry out their metabolic activity anaerobically (Brighton and Heppenstall, 1971). Proteoglycan aggregation was found to be higher at low oxygen tensions of 3% in chondrocytes cultured in vitro compared to culture in 21% oxygen tension (Clark et al., 1991). Under physiologic conditions, cartilage exists under low oxygen tension conditions, sometimes with oxygen tension as low as < 1%. In vitro, studies show that low oxygen tension has a beneficial effect on chondrocyte proliferation and matrix deposition. Bovine cartilage explants cultured under oxygen tension of 6% showed the same level of GAG synthesis as in fresh tissue, when cultured over 7 days with 20% fetal calf serum (Ysart and Mason, 1994). Chondrocytes cultured in monolayers showed an increase in matrix synthesis in the first two weeks of culture followed by no net increase in matrix production in a 20 day culture at 1% oxygen tension (Murphy and Sambanis, 2001b). A low oxygen tension of 5% also helps to restore the key phenotypic markers of passaged chondrocytes in alginate cultures (Murphy and Sambanis, 2001a). Thus low oxygen tension is an important parameter for bioprocessing conditions for in vitro chondrogenesis and cartilage construct development.

ATP is the universal energy currency in all cells and its formation is coupled to the oxidation of carbon rich energy sources. Glycolysis is the energy conversion pathway in many cells. Glycolysis is the pathway used to convert one molecule of glucose to two molecules of pyruvate. In anaerobic conditions, or in

aerobic organisms undergoing very high rates of glycolysis, the pyruvate is converted to lactate. The pH falls when large quantities of lactate are produced. Chondrocytes undergo a combination of aerobic and anaerobic metabolic pathways. During hypoxia, the cells shift to a primarily glycolytic mode for their energy needs. Thus, when chondrocytes are cultured in low oxygen tension, lactate production increases in monolayer culture. Dynamic changes in the oxygen tension environment constitute a potential signaling mechanism for the regulation of the expression and activation of redox signaling molecules and inflammatory cytokines (Vittur et al., 1994; Kuettner et al., 1989).

Biochemical changes associated with low oxygen tension in chondrocytes are poorly understood and the reports on the effect of low oxygen tension on chondrocytes are contradictory. Imposition of a hypoxic environment on cultured chondrocytes causes an increase in glucose consumption and lactate synthesis. Low oxygen tension plays an important role in regulating ECM synthesis in cartilage tissue engineering. However, the mechanisms of action of hypoxia are not clearly known in three dimensional chondrocyte cultures.

The variation in oxygen tension regulates the functioning of transcription factors like hypoxia inducible factor -1α (HIF-1α) (Cramer et al., 2004; Coimbra et al., 2004). HIF-1 is a transcription factor that binds specifically in hypoxia to a hypoxia response element in the promoter or enhancer region of the various hypoxia inducible genes. These genes include erythropoietin (EPO), vascular endothelial growth factor, glucose transporters and glycolytic enzymes. HIF-1α null chondrocytes were unable to maintain ATP levels in hypoxic

microenvironments, indicating that this factor is essential for the regulation of chondrocyte metabolism. HIF-1α also affects the extracellular matrix synthesis by chondrocytes. The synthesis of type II collagen is decreased under low oxygen conditions by chondrocytes lacking HIF-1α (Cramer et al., 2004). Therefore, it maybe possible that in vitro, HIF-1α may play an important role in chondrocyte metabolism.

Mechanical loading and fluid induced shear stress

Chondrocytes exhibit a distinct sensitivity to static and compressive loading. This response to loading is observed in both explants and in vitro engineered tissue and therefore is relevant to engineered cartilage (Torzilli et al., 1997). Mechanical loading alters cartilage metabolism. Physical forces such as compression, fluid flow, and hydrostatic flow have been implicated in the regulation of matrix metabolism in articular cartilage in vivo, as well as in cartilage explants and tissue engineered cartilage constructs (Freed et al., 1994b; Jin et al., 2001). Studies have been conducted to compare static vs. dynamic compression on cartilage explants (Davisson et al., 2002a; Wu et al., 1999). Dynamic compression increases the amount of matrix produced in cartilage constructs in vitro whereas static compression decreases the matrix content (Davisson et al., 2002a). Dynamic compression also stimulates production of matrix in cartilage explants. For engineered tissue, static compression decreases chondrocyte metabolism (Jin et al., 2001). Based on these results, the response of chondrocytes seems to be favored towards a dynamic loading regimen.

The functionality of the cartilage construct depends mainly on its mechanical properties. Correlation of fluid flow through and around the construct with matrix synthesis and matrix loss during dynamic compression protocols suggests that fluid flow is an important stimulatory signal. Fluid flow modulates cellular alignment and shape. Also, fluid flow through the construct increases the synthesis and accumulation of sulfated GAGs and collagens leading to improved compressive properties of the constructs. Fluid flow direction may play an as yet unidentified role in the fibrillar arrangement of collagen type II and proteoglycan fibers. The kinetics of matrix production and cellular proliferation is also affected by culture during fluid flow. These effects may be modulated by the intracellular calcium signaling and such changes are associated with cellular proliferation and matrix synthesis. Thus, there is a need for a well-defined fluid flow regime in both the radial and axial directions within the construct to promote matrix synthesis and deposition. Intermittent compression or shear increases production of ECM and the construct cultured under such conditions was significantly thicker compared to unstimulated controls (Davisson et al., 2002a).

These data indicate that the use of varying shear stress over the culture period as opposed to steady shear is a potentially powerful way to stimulate chondrocytes and enhance extracellular matrix deposition.

Growth factors

Growth factors like the transforming growth factor (TGF-β), insulin like growth factor (IGF), and bone morphogenetic proteins are some of the growth factors that have been used in cartilage tissue engineering to promote

differentiation, proliferation and extracellular matrix deposition. TGF-β1 has been used in chondrogeneic differentiation from mesenchymal progenitor cells (Barry et al., 2001; Fukumoto et al., 2003). In vitro, studies on cartilage explants and engineered tissue on the effect of TGF-β show that this growth factor increases the rate of proteoglycan synthesis. Also, TGF-β is capable of redifferentiating phenotypically altered chondrocytes in osteoarthritic cartilage (Grimaud et al., 2002). TGF- β1 used at a concentration of 30 ng/ml increased construct collagen content by 15% over 4 weeks in a spinner flask bioreactor configuration (Blunk et al., 2002). IGF-1 independently and in combination with mechanical loading increased construct extracellular matrix deposition in tissue engineered cartilage constructs (Gooch et al., 2001a). Bone morphogenetic proteins 2, 12, and 13 used in the concentration range of 1-100 ng/ml increased both GAG and collagen deposition in cartilage constructs (Blunk et al., 2002). The combination of IGF-1 (50 ng/ml) and TGF- β1 (30 ng/ml) increased construct GAG and collagen content by 63% and 67% respectively as compared to controls (Blunk et al., 2002). Thus, growth factors by themselves and in combination with mechanical loading can be powerful regulators of chondrocyte proliferation and extracellular matrix deposition in tissue engineering.

Thus, bioreactors can be effectively used to impart the various mechanical and biochemical forces required to grow implantable cartilage constructs. One of the issues that needs to be addressed is fundamental studies that help identify combinations of cells, scaffolds, nutrient and hydrodynamic loading conditions that will lead to tissue suitable for implantation. Current technology focuses

primarily on growing one or a small number of constructs under conditions that are not well defined. Under these circumstances it is difficult to quantitatively relate the bioreactor operating conditions to construct ultrastructure and material properties. Bioreactors such as the spinner flask and rotating wall vessel bioreactors do not provide a uniform flow environment for tissue growth. These bioreactors also exhibit poor fluid volume utilization and produce heterogeneous tissue constructs. The concentric cylinder bioreactor for the growth of cartilage constructs provides a uniform hydrodynamic and nutrient environment for the constructs (Saini, 2002; Williams et al., 2002). There is a need to identify specific conditions that enhance extracellular matrix deposition in the construct.

Little quantitative data exist to predict how bioreactor conditions will enhance tissue development. The complex mechanical environment cartilage experiences in vivo dictates the metabolic events within the tissue and has prompted much work in defining how mechanical factors stimulate chondrocyte cell function in vitro. Although mechanical and biochemical stimulation enhances ECM synthesis in bioreactors, the rate of matrix deposition decreases with time (Saini and Wick, 2003; Gooch et al., 2001b) leading to constructs with inferior mechanical properties.

Motivation for Current Research

Based on these observations, the current research seeks to identify the effect of specific bioprocessing conditions on construct extracellular matrix content and material properties. Because multiple factors affect cartilage tissue engineering, this work focuses on two primary chondrocyte modulators - fluid-

induced shear stress and low oxygen tension. Specifically, the effect of varying hydrodynamic loading is studied under two loading schedules – increasing hydrodynamic loading and decreasing hydrodynamic loading. These loading schedules are detailed in Table 1. The combination of decreasing hydrodynamic loading with low oxygen tension (5%) and TGF-β1 is explored. These studies are carried out in the concentric cylinder bioreactor. In order to incorporate multiple fluid flow regimes through the construct, the perfusion concentric cylinder bioreactor is designed. This bioreactor allows for shear stress on construct surface and perfusion flow through the construct. Short term and initial long term construct studies have been carried out in this bioreactor to study the effects of multiple fluid flow regimes.

A challenge for cartilage tissue engineering is increasing mechanical strength of the constructs. While the composition (chondrocytes, proteoglycan and collagen) of cartilage constructs from bioreactors is similar to native cartilage (Kuettner et al., 1991), tissue-engineered cartilage grown in bioreactors is relatively weak, with mechanical properties approximately ~10-25% of native cartilage (Vunjak-Novakovic et al., 1999; Freed et al., 1997; Martin et al., 2000; Noel et al., 2002; Vunjak-Novakovic et al., 2002), except when bioreactor culture time is 7 months (Martin et al., 2000). Since greater matrix accumulation enhances construct mechanical properties (Martin et al., 2000), a quantitative understanding of how bioreactor environment promotes cartilage development is important. In this work, systematic manipulation of select hydrodynamic and

nutrient environments in order to increase construct mechanical properties has been addressed.

Table 1: Hydrodynamic loading regimens studied in the concentric cylinder bioreactor

Loading regimen	0-12 days	13-24 days	25-35 days
Decreasing hydrodynamic loading	76 rpm	38 rpm	19 rpm
Increasing hydrodynamic loading	19 rpm	38 rpm	76 rpm

MATERIALS AND METHODS

All chemicals are obtained from Sigma Chemical Company (St. Louis, MO) and tissue culture supplies from VWR. Fetal bovine serum was obtained from Atlanta Biologicals Ltd (GA).

Tissue Digestion And Cell Isolation

Knee joints from freshly slaughtered male calves 2-4 weeks old were obtained from either Lampire Biologicals (Pipersville, PA) or from Research 87 (Marlborough, MA) within 24 hours of slaughter. The joints were shipped on ice overnight to the Georgia Institute of Technology. Fresh articular cartilage from the femoral-patellar grooves was obtained under aseptic conditions as described earlier (Freed et al., 1993a; Freed et al., 1994a). The joint was cleaned, and the exposed cartilage was washed in sterile phosphate buffered saline with EDTA (3.72 g/L) (PBE). Articular cartilage from the femoral patellar groove and femoral condyle was dissected using a sterile surgical scalpel. The pieces of tissue were collected in sterile petri-dishes containing PBE. The PBE was replaced with complete chondrocyte culture medium and the tissue was chopped into pieces of approximately 1 mm^3. Complete chondrocyte culture medium consisted of Dulbeco's Modified Eagle Medium (DMEM), containing glucose (4.5 mg/L) and glutamine (584 mg/L), 10% fetal bovine serum (FBS), ascorbic acid (100 μg/ml), non-essential amino acids (0.1mM), L-proline (0.4mM), 1% penicillin-

streptomycin, sodium bicarbonate (3.72g/L), 1% antimycotic-antibiotic, HEPES buffer (10 mM).

The tissue chunks were digested in collagenase type II (Worthington Biochemicals, PA) (activity 300U/mg) in DMEM (1.5 mg/ml) overnight in an incubator at 37°C and 5% CO_2. The resulting cell suspension was centrifuged in a centrifuge at 70g for 12 minutes. The supernatant solution was aspirated and sterile PBE was added to the 50 ml centrifuge tubes to wash the cells and remove residual collagenase. The cells were centrifuged three times for 12 minutes each with fresh changes of PBE for every wash. This was done to ensure that the cell viability was not harmed by long term exposure to collagenase. The cells were then suspended in 50 ml of complete DMEM and the cell pellet was broken by repeated pipeting using a 5 ml pipet. After a uniform cell suspension was obtained, 100 µl of this cell suspension was taken in a 1.5 ml Eppendorf tube along with 400 µl of medium and 100 µl of Trypan Blue was added. The cell number was obtained by using a hemacytometer using Trypan Blue exclusion method. Typical yield of chondrocytes was about 60 million cells per gm wet weight of native tissue.

Scaffold preparation and sterilization

Poly-L-Lactic acid crystals with a weight averaged molecular weight of 100,000 were obtained from Polysciences Inc (Warrington, PA). Scaffolds were prepared by porogen leaching using sodium chloride crystals as previously described (Saini and Wick, 2003; Mikos et al., 1994). PLLA crystals (0.5 gm) were dissolved in 5-7 ml methylene chloride (Fisher Scientific, Pittsburgh, PA).

The crystals were allowed to dissolve completely in methylene chloride and this took about 20-30 minutes. Sodium chloride (4.5 gm) with crystal diameter 106-150 μm was poured into a 50 mm Teflon dish and the polymer solution was added to it and mixed well. The polymer casts were allowed to set for about 48 hours. The cast polymer was then placed in a beaker with deionized (dI) water and sodium chloride was leached out into solution. The salt leaching was done for about 48 hours with multiple changes of deionized water. The scaffolds were punched into 10 mm diameter disks using a biopsy punch. The polymer scaffolds were allowed to dry and mounted onto thin plastic shims using a very small amount of inert silicone adhesive. Scaffolds were sterilized by three washes for 15 minutes each in 70% ethanol and three washes with sterile deionized water. After this treatment, the scaffolds were treated as sterile. Just prior to mounting the scaffolds on the bioreactor, they were exposed to ultraviolet light for 30 minutes. For all bioreactor studies, scaffolds were 1 cm in diameter, ~ 2 mm thick and 90% porous.

Concentric cylinder bioreactor

The concentric cylinder bioreactor was designed in our lab by Dr. Sunil Saini, a former graduate student at Georgia Institute of Technology (Saini, 2002; Saini and Wick, 2003; Saini and Wick, 2004; Williams et al., 2002). This bioreactor was used to perform the studies comparing the effects of different loading regimens and low oxygen tension on cartilage construct development. The bioreactor design is described in detail in the background.

Construct growth studies

Different conditions were examined in the concentric cylinder bioreactor to study at the effect of hydrodynamic loading caused by fluid-induced shear stress, and the effect of combining low oxygen tension at 5% with hydrodynamic loading. The experiments carried out for this are shown in Table 2.

Table 2: List of Experiments

Experiment name	Comparison	Culture Time	Number of bioreactors	Construct harvest days	Number of experiments
Low oxygen tension at steady shear	Rotation 38 rpm 20% vs. 38 rpm 5% O_2	35 days	2	12, 24, 35	2
Decreasing hydrodynamic loading	Steady shear at 76 rpm vs. DHL at 76, 38, 19 rpm	35 days	2	12, 24, 35	4
Increasing hydrodynamic loading	Steady shear at 19 rpm vs. IHL at 19, 38, 76 rpm	35 days	2	12, 24, 35	3
Decreasing hydrodynamic loading with low oxygen tension	DHL and 20% O_2 vs. DHL and 5% O_2	35 days	2	12, 24, 35	4
Effect of TGF-β1	No TGF-β1 vs 0.2 ng/ml TGF-β1	24 days	2	12, 24	1
Effect of TGF- β1 + DHL	DHL vs DHL + TGF-β1 0.2 ng/ml	35 days	2	12, 24, 35	4
Perfusion bioreactor seeding study	150 X 10^6 cells for 16 constructs	4 days	1	2, 3, 4	4
Short term construct development	Perfusion from day 4 to day 14 (10 ml/min)	14 days	1	4, 7, 11, 14	4
Long term construct development	Perfusion from day 4 to day 35 (10 ml/min)	35 days	1	4, 12, 20, 28, 35	1

Perfusion Concentric Cylinder Bioreactor

The primary goal of the perfusion shear flow bioreactor is to incorporate two flows through the construct. The objective of doing this is to induce cell alignment through the thickness of the tissue and obtain cartilage with architecture similar to that of native cartilage.

Design and development

The perfusion concentric bioreactor incorporates homogenous fluid flow environment external to the construct with fluid perfusion through the construct (Figure 1). This bioreactor incorporates media flow through porous constructs to increase contact of the media with the scaffold and expose the interior of the construct to flow for better cell seeding and matrix deposition. This bioreactor is also capable of promoting cell alignment within the construct and can be used to induce anisotropy in the engineered tissue similar to native tissue. The perfusion concentric bioreactor consists of an outer polypropylene cup and an inner central tube designed to perfuse constructs. The outer cup is held on a polypropylene base which is connected to a motor. This is the same as in the concentric cylinder bioreactor (Figure 4). Figure 5 shows the inner tube with the construct housing.

The entire inner perfusion tube is made of polycarbonate to facilitate visual observation of the fluid flow. The inner tube is attached to an aluminum support bar with provisions to allow a fluid flow circuit from the media reservoir to the inner tube. Fluid is pumped in through the inner tube by means of a roller pump. A loose fitting Teflon cap is provided to cover the glass cup to prevent possible

contamination. A working prototype perfusion concentric bioreactor has been built with 2 rows of construct holders (Figure 5). The simple scalable geometry allows for easy incorporation of more construct holders on the central tube for scale-up purposes. An overflow reservoir collects the media in the cup and provides for media circulation through the construct. This overflow is designed to maintain the fluid level in the glass cup and allows for constant replenishment of fresh media. Media is pumped in through the constructs at a controlled rate to facilitate construct seeding and tissue growth.

The construct holder (Figure 6) is designed so that most of the construct surface is exposed to fluid flow. Tissue constructs must be fixed in space to have controlled mechanical stimulation but fluid flow through the constructs must not be constrained. The design of the construct holder is such that it allows for construct development with minimal contact of the polymer scaffold to any other surface.

The construct holder built for the prototype has an outer diameter of 1.25 cm and inner diameter of 1 cm. The constructs used in the preliminary studies are 1cm in diameter and 2 mm thick. The mouth of the construct holder has a gradual narrow to wide progression in terms of inner diameter. Very small nylon set screws are positioned at the end of the holder. The set screws restrain the constructs from flowing out into the outer cup without causing physical damage to the constructs. The narrow inner diameter of the construct holder tube and the set screws essentially hold the constructs in place with minimal contact on the faces of the scaffold. This way, most of the construct surface area is available

for perfusion flow, cell seeding and matrix deposition. Culture medium is constantly pumped through the central tube which plays the role of a hub for all the construct holders. As the medium level rises, the fluid is continuously pumped out from the overflow reservoir. A photograph of the inner tube from the bioreactor prototype is shown in Figure 7.

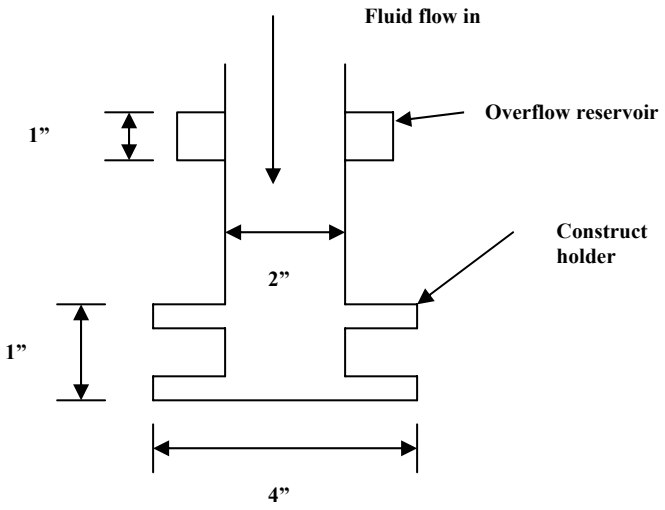


Figure 5: Schematic of the perfusion concentric cylinder bioreactor. Shown here is the outer cup and the inner perfusion tube with construct holders.

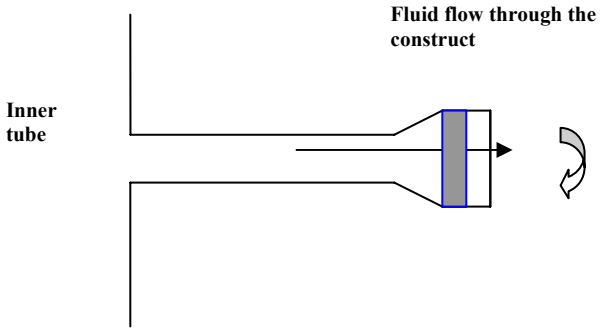


Figure 6: Construct holders. The constructs are held using nylon retainer clips or set screws.

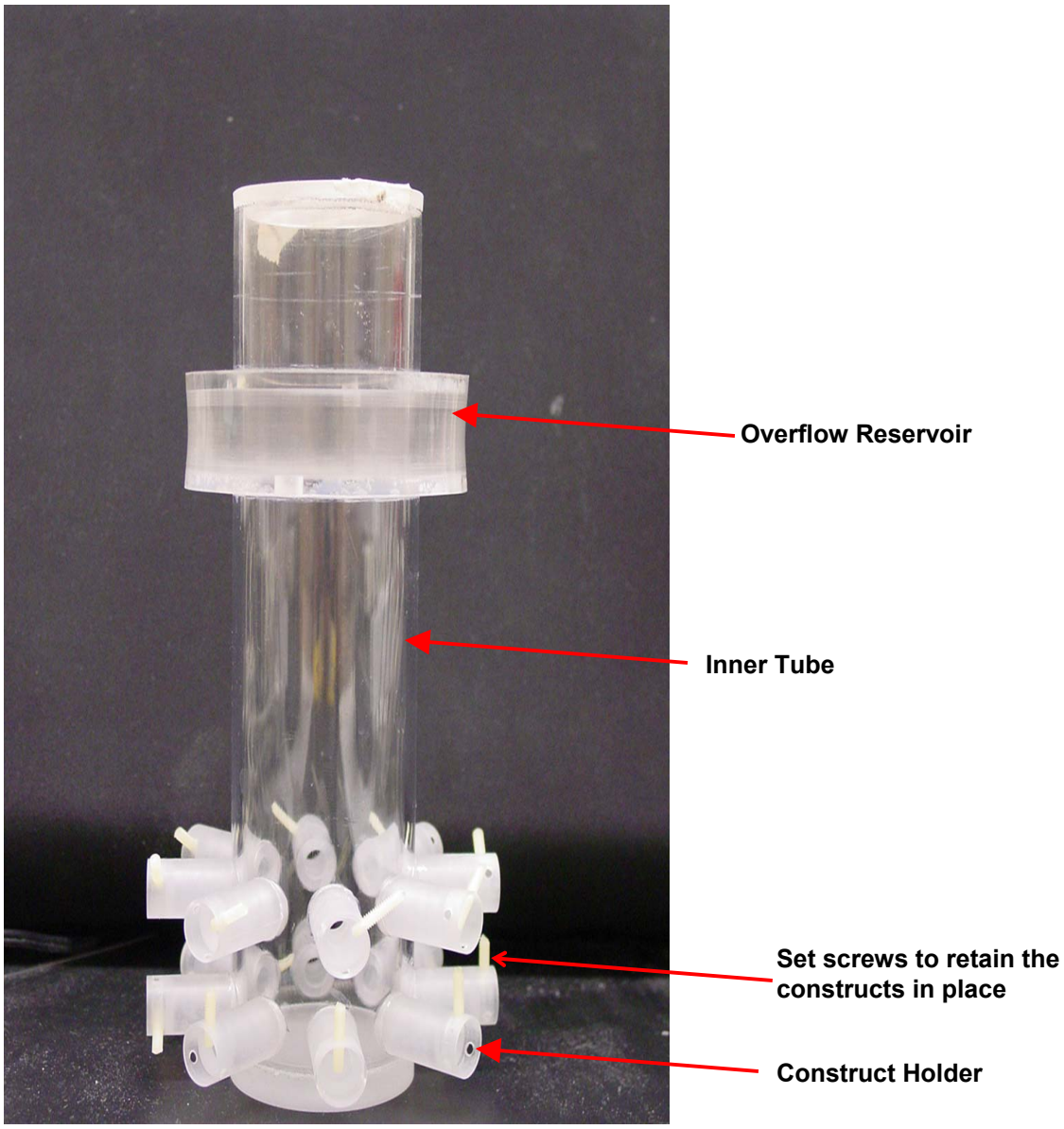


Figure 7: Photograph of the prototype inner tube built for the perfusion shear flow bioreactor. The inner tube, construct holder, overflow reservoir and set screws used to retain the constructs in place are marked by the red arrows.

The gap between the outer glass cup and the inner cylinder is increased to 10mm in this bioreactor configuration. Previous studies show that maintaining a 3 mm gap between the inner bob and the outer glass cup in the concentric cylinder bioreactor did not cause any oxygen deficiency in the constructs during the growth period. The perfusion system design also allows for increased oxygen dissolution in the media. Thus, this system continues to provide required oxygen supply to the constructs.

The constant surface shear in the concentric cylinder bioreactor is because of the uniform gap width between the concentric cylinder. The gap width is not uniform in the perfusion design. However, because of the outer cup rotation, the construct surface experiences shear stress. It is assumed that the all the constructs experience surface shear stress in the same range because they are located at the same distance from the outer cup.

The perfusion system has a high potential for scale up because of its inherent design. The ability of the cup to contain a large volume of media and the length of the central tube allow for easy scalability of the bioreactor. The number of construct holders can be increased along the length of the inner tube to facilitate increase in the volumetric productivity. Availability of nutrients is not a limiting concern because the fluid volume is large in the bioreactor and perfusion increases nutrient supply to the interior of the constructs. Increasing the capacity of the bioreactor only requires linear scale-up and not a series of complex calculations.

Perfusion flow, shear stress, hydrodynamic loading, increased oxygen supply and a large scale-up potential make this device capable of incorporating the necessary physical factors required for chondrocyte proliferation, matrix deposition and tissue maturation. Previous studies have shown that each of the aforementioned factors when individually applied showed a positive effect on chondrogenesis and matrix production. The perfusion concentric cylinder bioreactor allows for simultaneous application of these physical parameters for the optimal tissue engineering of cartilage constructs.

Construct growth studies in the perfusion bioreactor were carried out to evaluate cell seeding efficiency, cell viability in the bioreactor environment, short term construct development and preliminary long term construct development. These studies are outlined in Table 2

Bioreactor Set-up

Two days prior to start of the experiment, the bioreactor outer cup and inner bob or tube were treated with Sigmacote® to form a thin, inert silicone coating on the bioreactor components to prevent cell adhesion. The Sigmacote® solution was allowed to evaporate overnight. The bioreactor components were then washed with soap and water and allowed to air dry prior to steam sterilization in an autoclave. Steam sterilization was carried out at 121°C and 15 psig for 20 minutes. After sterilization, the bioreactor parts were assembled aseptically in the laminar flow hood.

For all construct growth studies in the concentric cylinder bioreactor, 16 scaffolds were used. These scaffolds were attached to the inner bob in a regular

staggered array pattern in two rows. The constructs were pasted on a thin plastic shim and mounted to the inner bob by means of inert silicone adhesive. In the perfusion bioreactor, the sterile scaffolds were placed in the construct holders and the set screws used to restrain the constructs in place were tightened using sterile forceps.

Bioreactor Seeding and Feeding

Concentric Cylinder Bioreactor

The bioreactor mount was cleaned and sprayed with 70% ethanol and then placed in a 37°C, 5% CO_2 incubator. After the chondrocyte cell suspension was prepared and the cell concentration was determined, the volume of cell suspension containing 100 X 10^6 cells was added to the bioreactor cup. Then complete DMEM was added to bring up the total volume to 62 ml. The solution was made uniform by a series of pipetting steps using a 5 ml pipette. The inner bob was lowered carefully into the cup. The bioreactor was then placed on the rotating base in the incubator. The inner bob was stabilized by fastening it on to the support arms with set screws. These steps were repeated for each bioreactor. The motor speed controller was fit with a timer device that allowed for automated change of direction of rotation every 12 hours.

After 48 hours, 31 ml of the culture medium was replaced. After 4 days from seeding, the entire 62 ml of medium was replaced. From then on, the bioreactor was fed every 48 hours with 31 ml media change. To replenish culture medium, the bioreactor was moved to the laminar flow hood. The inner

bob was removed carefully and placed in a sterile 150 mm petri dish. The designated amount of cell culture medium was removed from the bioreactor cup, and 1 ml was saved in a labeled Eppendorf tube for further analysis. The required amount of fresh DMEM at 37°C was added to the cup and the inner bob was lowered into the cup.

Perfusion Bioreactor

For the perfusion bioreactor, the seeding was done as 150 X 10^6 cells per bioreactor because of the increased volume in the bioreactor. One hundred and fifty million cells were added to 500 ml of culture medium in the outer cup. Because of the hollow inner tube and the increased gap between the outer cup and the inner tube, the volume of culture medium added to the perfusion bioreactor was large. At least 400 ml of culture medium was required to submerge the two rows of constructs. After addition of culture medium, the inner tube was slid into the outer cup. The seeding phase for the perfusion bioreactor was carried out for 4 days without perfusion. Perfusing the cell suspension through a roller pump would cause the death of most of the cells. Therefore, seeding was carried out through outer cup rotation maintained at 38 rpm in all studies. After 4 days of seeding, the complete medium in the cup was changed and replaced with fresh medium. Fresh medium was added to the reservoir. Perfusion was carried out at 10 ml/min and recirculated. For the 14 day experiments, the medium was replaced on days 4, 7 and 11 after initiation of the experiment. For the 35 day experiment, the bioreactor was fed at 3-4 day

intervals. All the components were treated with Sigmacote® to prevent cell adhesion to any bioreactor surface.

Once cell seeding was completed, the remaining cell suspension was drawn out of the bioreactor to check for cell seeding efficiency and to remove the dead and/or unattached cells. The cup was refilled with enough culture medium to submerge the constructs and then fluid was pumped through the inner tube. To complete the loop, fluid was continually pumped out of the overflow thereby removing the excess culture medium. The medium in the reservoir was changed periodically. Constructs were harvested at different time points for biochemical analyses.

Construct Harvest

Construct harvest schedule depended on the experiment design (Table 2). All constructs harvested were washed in PBE to rid them of serum components and stored in Eppendorf tubes at -20°C until further analysis. Thirty five day samples were used for mechanical testing. These samples were washed in PBE and then frozen at -20°C, in PBE containing 1X protease inhibitor cocktail. The cocktail would prevent any degradation of extracellular matrix components while in storage.

Construct Analysis

For biochemical analysis, the entire construct was digested in papain and aliquots were used for different assays.

Construct Digestion

Harvested constructs were frozen, dried in a vacufuge and digested in papain enzyme solution. The papain solution was prepared by dissolving 500 µl of papain solution in 100 ml of PBE at an activity of 35 U/mg of papain (Worthington Biochemical Corporation, Lakewood, NJ). 1 ml of this solution was added to each construct. The Eppendorf tube was then placed in a high temperature water bath at 56°C and the entire construct was digested overnight. The digested samples were stored at -20°C until further analysis.

Construct DNA Determination

The DNA content in the constructs was determined using a fluorescent dye. The quantitative Picogreen DNA determination kit was obtained from Molecular Probes (Eugene, OR). The kit was used in accordance with the accompanying procedure. The dilution buffer provided with the kit was a 20 X TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 7.5). This buffer was diluted to 1X. Calf thymus DNA standard at 100 µg/ml was provided. Dilutions of the standard were made to obtain final concentrations as shown in Table 3. The Picogreen reagent was prepared by adding 0.1 ml of Picogreen dye to 19.9 ml of 1X TE buffer. The samples usually had to be diluted in the ratio 1:20 to minimize noise in the assay and obtain accurate measurements. The assay was carried out in a 96 well black microplate. 100 µl of sample was added to the well and 100 µl of freshly prepared dye was added. The plate was covered in aluminum foil and incubated at room temperature for 2-5 minutes. The plate was read in a fluorescence plate

reader at excitation of 480 nm and emission of 520 nm. The resulting amount of DNA was calculated by interpolating on the standard curve. The cell number was calculated by using 7.7 pg DNA per chondrocyte (Kim et al., 1988).

Table 3: DNA standards for Picogreen assay

Volume (μl) of 2 μg/ml DNA stock	Volume (μl) of TE buffer	Volume (μl) of Picogreen reagent	Final DNA concentration (ng/ml)
0	100	100	0
0.1	99.9	100	1
1	99	100	10
10	90	100	100
30	70	100	300
50	50	100	500
80	20	100	800
100	0	100	1000

Table 4: GAG assay standards

Chondroitin Sulfate	Working Solution	DI water
1 µg	100 µl	900 µl
2 µg	200 µl	800 µl
3 µg	300 µl	700 µl
4 µg	400 µl	600 µl
5 µg	500 µl	500 µl
6 µg	600 µl	400 µl
7 µg	700 µl	300 µl
8 µg	800 µl	200 µl
9 µg	900 µl	100 µl
10 µg	1000 µl	0 µl

Construct GAG determination

The amount of sulfated GAG present in the constructs was determined by the method of Farndale (Farndale et al., 1982). Chondroitin sulfate (CS) standards were prepared by dissolving bovine CS in PBE to obtain a stock solution of 50 mg/ml. A working solution of 100 µg/ml was prepared by diluting the stock solution. Standards were prepared as shown in table 4.

The working DMMB dye solution was prepared by mixing glycine (40.5 mM), NaCl (40.5mM), and hydrochloric acid (9.5 mM). After dissolution, 16 mg DMMB was added to 1 liter of this solution, pH was adjusted to 3 and the dye was stored in the dark at room temperature. The assay was carried out in a plate reader in a 96 well plate format. Eight microliters of sample or standard and 200 µl of dye were added to each well and allowed to incubate at room temperature for 2 minutes. The plate was then read at a wavelength of 525 nm. Negative controls used were PLLA constructs with no cells and positive controls were native cartilage samples. The standard curve was plotted and values of GAG in the sample were obtained by linear interpolation.

Construct Collagen Content

The amount of collagen present in the constructs was determined by measuring the amount of hydroxyproline in the sample and multiplying by a factor of 9 to obtain total collagen (Woessner, 1961). Hydroxyproline was used as a standard for the assay. Briefly, a working solution of 1 mg/ml of hydroxyproline was made and subsequent standards in the range of 0-50 µg were prepared. Aliquots of samples (under 10 µg/ml) and standards were prepared in glass

tubes and 2N NaOH was added to this solution. Samples and standards were diluted in an acetate buffer containing 120 mg/ml sodium acetate, 46 mg/ml citric acid, and 34 mg/ml sodium hydroxide with pH 6.5. The total volume was brought up to 50 μl. The samples were then hydrolyzed by autoclaving at 121°C and 15 psig. A solution of Chloramine-T was prepared by dissolving 1.27g of Chloramine-T in 20 ml n-propanol and making the solution up to 100 ml using dI water. All solutions were prepared just before use. Four hundred and fifty microliters of Chloramine-T was added to the hydrolyzed sample and incubated for 25 minutes at room temperature. The Ehrlich's reagent was prepared while the sample was incubated with Chloramine-T. The reagent consisted of 15 g of p-diaminobenzaldehyde in 100 ml of a 2:1 solution (v/v) of n-propanol and perchloric acid. Five hundred microliters of the reagent was added to the sample containing Chloramine-T and incubated at 65°C for 25 minutes. The samples were transferred to cuvettes and read in a spectrophotometer at 540 nm. The hydroxyproline content in the sample was determined by linear interpolation of the standard curve.

Collagen type II ELISA

To detect the presence of collagen types I and II, a semi quantitative enzyme linked immunosorbent assay (ELISA) was performed. The total amount of protein in the construct was measured by the method of Bradford using a protein determination kit (BioRad, Hercules, CA). The digestate was diluted in 1X phosphate buffered saline (PBS), pH 7.2 to a final protein concentration of approximately 20 μg/ml. Fifty microliters of the sample was coated on a 96 well

polystyrene plate. Standards used were bovine collagen type II and bovine collagen type I. After coating the plates for 1 hour at 37°C, non specific protein blocking was performed by the addition of a 1% bovine serum albumin solution in PBS (1%BSA/PBS). After blocking for 30 minutes, the blocking solution was removed from the wells and primary antibody was added. To detect background generated due to secondary antibody binding, each sample was added in two wells in the 96 well plate. One well was exposed to both primary and secondary antibodies while the control well was exposed only to secondary antibody. Primary antibodies for type II collagen and procollagen type I were obtained from the Development Studies Hybridoma Bank (University of Iowa). Primary antibodies were diluted 1:15 and secondary antibody (goat-anti-mouse) was diluted 1:1000. All antibody dilutions were in 1% BSA/PBS. After primary antibody incubation for an hour at room temperature, the wells were washed with 0.05% Tween-20 in PBS (PBS-T) three times. The secondary antibody was added and incubated for an hour at room temperature. After washing the excess secondary antibody, the antibody conjugate was detected by means of o-phenylenediamine dihydrochloride peroxidase substrate (Perlmann and Perlmann, 1994). The optical density was read using a spectrophotometer at 450 nm and amount of type II and type I collagen were determined by using a log-log standard curve.

Histology

35 day constructs were washed in PBE immediately after harvest. The constructs were fixed in 10% neutral buffered formalin and stored at 4°C for up to a week. Post-fixation, the samples were incubated in 15% sucrose overnight. This allowed the sucrose solution to penetrate the pores of the tissue and replace the formalin. Then the constructs were embedded in an optimal cutting temperature medium containing 10% poly vinyl alcohol. Sections 7-μm through the construct thickness were made using a cryostat on to plain glass slides. The sections were stored at -80°C until ready for staining.

Before staining, the slides were air-dried for 20 minutes. The slides were then fixed in acetone for 5 minutes. The slides were air-dried again to remove any traces of acetone. The slides were stained for cell nuclei (Hematoxylin & Eosin), GAG (safranin-O) and collagen (Masson's trichrome). Immunofluoresence was used to detect the presence of collagen type II.

Confocal Microscopy

The cell viability inside constructs was assessed after 4 days in the seeding efficiency studies using the live/dead assay kit (Molecular Probes Inc., OR). The protocol accompanying the kit was followed to stain the cells with Calcein-AM and ethidium homodimer to visualize live and dead cells. 20 μL of the supplied 2 mM ethidium homodimer stock solution was added to 10 mL of sterile, PBS, vortexing to ensure thorough mixing. To this solution, 5 μL of the supplied 4 mM calcein AM stock solution was added. The resulting working

solution had approximately 2 μM calcein AM and 4 μM ethidium homodimer. The working solution was added to freshly harvested construct rinsed briefly in PBS. The construct was then visualized using a confocal microscope.

Table 5: Hematoxylin and eosin protocol

Step	Procedure	# of washes	Duration
1	Xylene	3	3 min
2	100 % alcohol	3	2 min
3	95 % alcohol	2	2 min
4	70% alcohol	1	2 min
5	dI water	1	2 min
6	Hematoxylin	1	0.5-1 min
7	0.5% acid alcohol	1	1 dip
8	Water	1	1
9	Bluing reagent	1	30 sec
10	Water	1	1 min
11	95 % alcohol	1	1 min
12	1% alcoholic eosin	1	30 sec
13	95% alcohol	1	30 sec
14	100% alcohol	3	1 min
15	Xylene	3	1 min
16	Coverslip and mount media	--	--

Table 6: Safranin-O staining for glycosaminoglycan

Step	Procedure	# of washes	Duration
1	dI water	1	1 min
2	Weigert's hematoxylin	1	15 sec
3	Tap water	1	2 min
4	0.2% aq. fast green	1	1 min
5	1% acetic acid	1	3 sec
6	0.1% safranin-O	1	5 min
7	95% alcohol	1	1 min
8	Reagent alcohol	3	1 min
9	Xylene	2	1 min
10	Coverslip and mount media	--	--

Table 7: Masson's trichrome staining for collagen

Step	Procedure	# of washes	Duration
1	Rehydrate in dI water		
2	Bouin's solution (at 56°C)	1	1 hour
3	Tap water rinse	1	5 min
4	dI water rinse	1	2 min
5	Weigert's hematoxylin	1	1-2 min
6	Tap water	1	5 min
7	Biebrich's scarlet-acid fuchsin	1	5 min
8	dI water rinse	1	2 min
9	Phosphomolybdic-phosphotungstic acid	1	1 min
10	Aniline blue	1	5 min
11	1% acetic acid	1	5 min
12	95% alcohol	2	1 min
13	100% alcohol	2	1 min
14	Xylene	3	1 min
15	Coverslip and mount media	--	--

Table 8: Immunofluorescence for collagen II

Step	Procedure	# of washes	Duration
1	Rehydrate in 1 X PBS	2	5 min
2	Antigen retrieval using pepsin (37°C)	1	20 min
3	Wash in PBS	2	5 min
4	Block 1% BSA/PBS	1	20 min
5	1 antibody (1:1) diluted in 1%BSA/PBS	1	1 hour
6	Wash in PBS	2	5 min
7	Biotinylated 2 antibody diluted in 1% BSA/PBS (1:400)	1	30 min
8	Wash in PBS	2	5 min
9	Avidin D (in dark)	1	30 min
10	Wash in PBS	2	5 min
11	DAPI (12.5 µg/ml) (in dark)	1	5 min
12	Wash in PBS	2	5 min
13	Mount using Aquamount (store slides in dark)	--	--

Medium Analysis

Culture medium samples were saved from every feeding (every 48 hours) from the bioreactors. These samples were used to analyze for metabolic activity and the presence of soluble GAG and NO release in the medium.

Glucose Measurement

Medium glucose measurements were made using Trinder reagent. One vial of the reagent was dissolved in 100 ml of dI water. The assay was carried out in microwell format. One microliter of the medium sample was added to the well. Pre-made standards (100 mg/dl, 300 mg/dl and 800 mg/dl) were obtained from Sigma Chemicals. Two hundred microliters of the reagent was added to each well and the microplate was incubated at room temperature for 18 minutes. After incubation, the plate was read at 505 nm in a plate reader. The initial concentration of glucose in the medium was measured using fresh DMEM.

Lactate Measurement

Medium lactate levels were measured using a lactate reagent (Trinity Biotech, Ireland). The lactate reagent was prepared by dissolving 1 vial of the reagent in 10 ml of dI water. The assay was performed in a microwell format. Standards were obtained from Sigma Chemicals (20mg/dl, 80 mg/dl and 120 mg/dl). Two microliters of the media sample was added to the well and 200 µl of the reagent was added. The plate was incubated at room temperature for 10 min and then read in a plate reader 540 nm.

Soluble GAG Measurement

The soluble GAG in the spent medium was measured using the DMMB dye similar to the construct GAG content. Eight microliters of the medium was added to the microwell plate and 200 µl of the DMMB dye was added. The plate was incubated at room temperature for 2 minutes and read in a plate reader at 540 nm.

Nitric oxide Measurement

Nitric oxide is an unstable molecule and is converted to nitrate/nitrite in culture. The release of nitric oxide by the cells in the culture medium was measured by determining the nitrate/nitrite content in the medium. The Griess' reagent consists of 1% w/v sulfanilamide, 0.1% w/v napthylethylene diamine dihydrochloride and 2.5% phosphoric acid in dI water. Standards were prepared using sodium nitrite 0.01 µg -0.06 µg. Fifty microliters of sample was added to a microwell plate and then 50 µl of the reagent was added to the well. The plate was incubated at room temperature for 10 minutes, and read in a plate reader at 540 nm.

Mechanical Testing

Measurement of Dynamic Shear Modulus

Thirty five day samples for mechanical testing are frozen in PBE containing protease inhibitors solution at -20°C. Prior to testing, the samples were allowed to thaw for 30 minutes at room temperature. The construct shear modulus was

measured using a Bohlin CVO® rheometer. Two or three 4 mm biopsy punches were made from each construct. The sample thickness was measured and noted. A typical testing apparatus is shown in Figure 8. The sample was placed on the rheometer base plate and preloaded using an impermeable platen. The rheometer gap was set to 90% of the sample thickness to pre-load the sample. A small quantity of PBE with protease inhibitor cocktail was added to the base plate of the rheometer to maintain the sample hydration. After the sample had equilibrated, the oscillation test was carried out. The frequency sweep was carried out in the range of 0.01-1.0 Hz with logarithmic increase in the frequency. A typical test took about 15-20 minutes. The measuring system was then backed off and the sample was transferred back to the pre-labeled tube. This testing protocol causes very little change in the fluid volume of the construct and allows us to measure the flow independent viscoelastic behavior of cartilage.

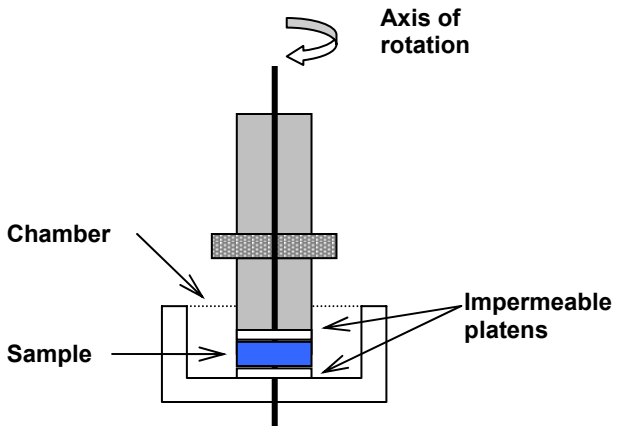


Figure 8: Schematic of the testing apparatus for the measurement of the shear modulus

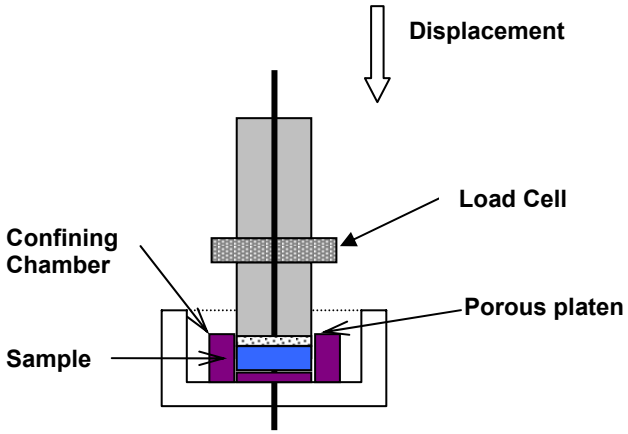


Figure 9: Schematic of confined compression chamber and indenter attached to load cell.

Confined compression testing

Confined compression testing was used to evaluate the flow dependent behavior of tissue engineered cartilage construct. A confined compression testing chamber 4 mm in diameter was used to measure the aggregate modulus of the constructs on an ELF machine (EnduraTEC, Minnetonka, MN). A schematic of the testing apparatus is shown in Figure 9. The testing apparatus is located in the mechanical testing core facility, in the IBB building. The construct thickness was measured and the construct was then placed in the confined compression chamber. A porous steel platen was placed on the construct and the construct was preloaded to 0.01 N. A stress relaxation test was initiated at strains of 5, 10, 15, 20%. The stress was allowed to reach equilibrium for 15 minutes. The sample was kept in a bath of PBE with protease inhibitor during the testing. The construct aggregate modulus was obtained by plotting equilibrium stress versus strain and calculating the slope. In most cases, the linear regression had a correlation coefficient greater than 0.9.

Statistical Analysis

An experiment consisted of seeding the required number of bioreactors with chondrocytes from the same animal. Different experiment within the same study used cells from different animals. For each data set, the values are presented as mean ± SEM for n constructs pooled from N different experiments. Individual data sets for each parameter were tested for normality or Gaussian distribution. If the data set was not normal, then a non-parametric test was carried out. For normally distributed data, analysis for statistical significance was

carried out using a general linear model analysis of variance (ANOVA) (Hayter, 1996). For the mechanical testing correlation with construct extracellular matrix content, non-linear regression methods are used. For the 35 day experiments, comparisons for determining statistical significance were carried out between conditions (for the pooled set of n constructs from N separate experiments) for the biochemical data for the 35 day constructs.

RESULTS

Many bioprocessing conditions affect the deposition of extracellular matrix in vitro. For cartilage tissue engineering, a continuing challenge has been to identify conditions that a) increase extracellular matrix synthesis, b) increase construct material properties, and c) provide constructs with an ultrastructure similar to native tissue. In this work, the main focus is on bioreactor design and operating conditions that address the factors listed above.

The effects of surface shear stress on construct development are well documented for the concentric cylinder bioreactor (Saini, 2002; Saini and Wick, 2003; Williams et al., 2002). Based on these studies and the varying loading that the joint experiences in vivo, experiments were carried out to determine the effect of variable hydrodynamic loading on construct surface through the culture period. This involved the study of construct biochemical composition under two loading regimens: increasing hydrodynamic loading (IHL) and decreasing hydrodynamic loading (DHL). The loading schedules are shown in Table 1 in the Background. Each loading regimen was studied separately with its own control. The steady control for DHL was a bioreactor operated at 76 rpm throughout the culture time and the steady control for IHL was a bioreactor operated at 19 rpm throughout the culture time.

Another well documented modulator of ECM synthesis in vitro is low oxygen tension (Murphy and Sambanis, 2001b; Murphy and Sambanis, 2001a). In the concentric cylinder bioreactor, constructs cultured at 5% oxygen tension

have higher GAG synthesis as compared to constructs cultured under 20% oxygen tension after 3 weeks in culture (Saini, 2002; Saini and Wick, 2003; Williams et al., 2002). This study was extended to 5 weeks in the current work. The combined effect of hydrodynamic loading and low oxygen tension was also studied. Some preliminary work on the modulation of constructs using TGF-β1 has also been done.

Effect of low oxygen tension at steady shear

The primary objective of this experiment was to determine the effect of low oxygen tension (5%) on cartilage constructs cultured for 35 days in the concentric cylinder bioreactor culture. The effect of 5% oxygen tension on construct development is documented for 22 days (Saini and Wick, 2004). Two bioreactors were operated at 38 rpm in an oxygen tension environment of either 20% or 5%. For this study, only two experiments were carried out (n=6; N=2).

The construct biochemical composition is shown in Figure 10. In either oxygen tension, robust cell proliferation was observed and the construct cell number did not vary largely over the culture period (Figure 10A). After 35 days, the constructs from the bioreactor operated in normoxia had 15 ±5 million chondrocytes per construct and constructs from the bioreactor operated in 5% oxygen tension had 24 ± 1.8 million cells per construct. The construct collagen content is shown in Figure 10C. The construct collagen content was similar in 20% and 5% oxygen tension on day 12 (0.99 ± 0.13 mg/construct and 0.8 ± 0.06 mg/construct respectively). On day 24, construct collagen content was higher in the 5% oxygen tension bioreactor (1.3 ± 0.15 mg/construct) as compared to 20%

oxygen tension (1.0 ± 0.15 mg/construct). On day 35, the construct collagen content was 1.45 ± 0.09 mg/construct in the 5% oxygen tension bioreactor, 1.5-fold more than the collagen content in the constructs cultured in the 20% oxygen tension bioreactor (0.95 ± 0.16 mg/construct). The construct GAG content in the 5% oxygen tension was also higher as compared to the construct GAG content in the 20% oxygen tension (Figure 10B) (5.16 ± 0.8 mg/construct as compared to 3.28 ± 0.37 mg/construct).

Thus, low oxygen tension culture increased construct extracellular matrix deposition over 35 days in bioreactor culture.

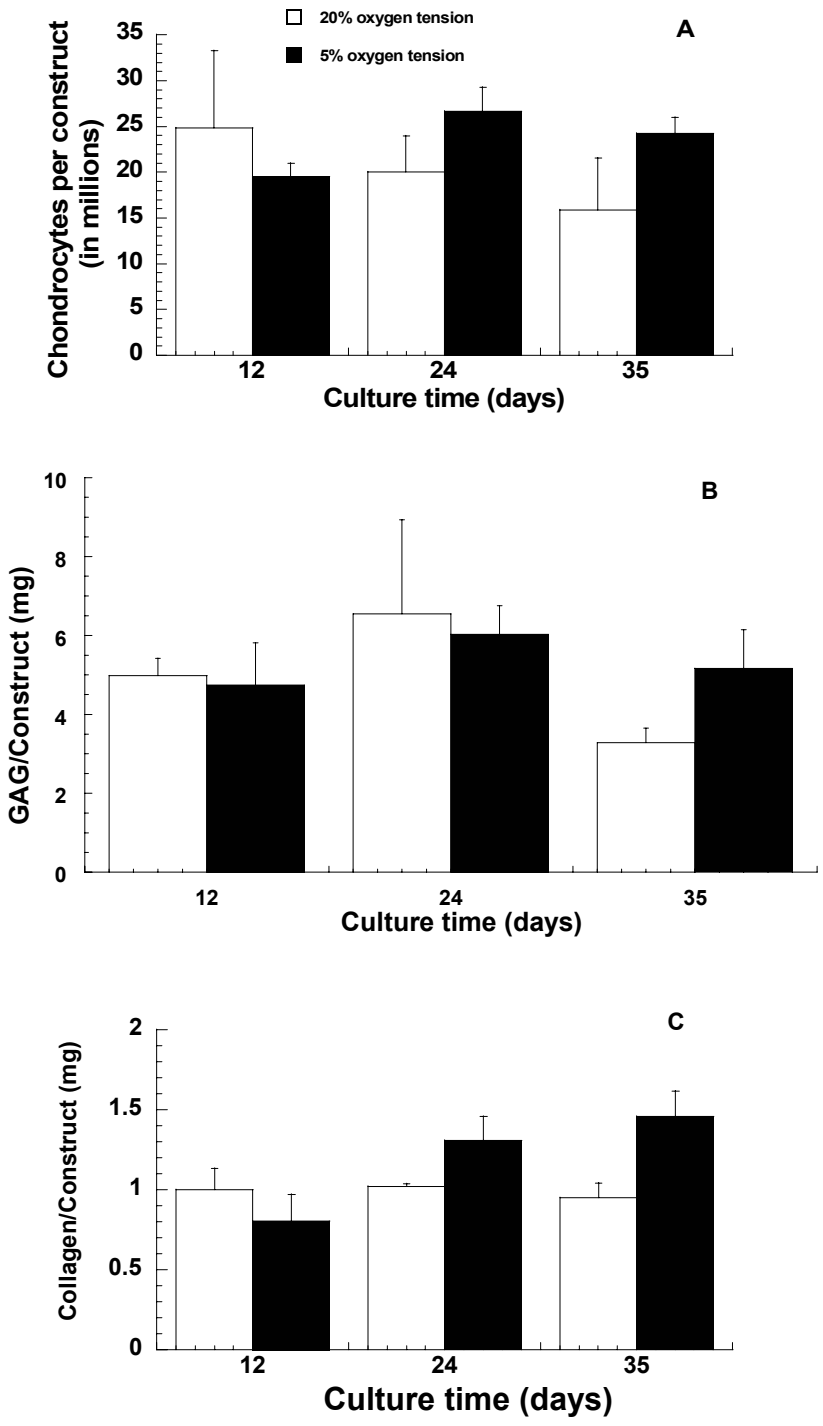


Figure 10: Effect of low oxygen tension on construct development. Data are mean ± SEM for n=6 constructs from N=2 experiments (A) Chondrocytes/construct (in millions), (B) GAG/construct (mg), (C) Collagen/Construct (mg).

Effect of decreasing hydrodynamic loading on construct development

The main objective of this study was to determine the effect of decreasing hydrodynamic loading (DHL) on construct biochemical composition. Two bioreactors were operated at 20% oxygen tension, one run at 76 rpm, the other bioreactor at DHL (Table 1, Background) for 35 days.

Chondrocytes exhibited approximately a 2-fold increase in cell number from the initial seeding number of 6.25 million cells per construct in the first 12 days of bioreactor culture. The constructs contained 11.6 ± 2.7 million cells/construct in 76 rpm as compared to 12.3 ± 2.7 million cells/construct in DHL (Figure 11A) on day 12. On day 24, the chondrocyte population in the 76 rpm regimen was 11.7 ± 2.7 million chondrocytes/construct as compared to 4.6 ± 1.2 million chondrocytes/construct in DHL (Figure 11A). On day 35, the construct cell numbers were 18.7 ± 7.7 million chondrocytes/construct in 76 rpm and 13.1 ± 2.7 million chondrocytes/construct in DHL (Figure 11A).

The construct GAG content was 3.7 ± 0.8 mg GAG/construct in 76 rpm as compared to 4.6 ± 0.6 mg GAG/construct (Figure 11B) on day 12. On day 24, the construct GAG content was similar in either bioreactor hydrodynamic loading, the values being 3.8 ± 0.9 mg GAG/construct and 4.3 ± 0.9 mg GAG/construct in 76 rpm and DHL respectively. At the end of culture on day 35, the construct GAG content was higher in DHL at 7.2 ± 0.5 mg GAG/construct as compared to 5.1 ± 0.5 mg GAG/construct in 76 rpm. This increase in construct GAG was statistically significant (* $p<0.05$).

On day 12, the construct collagen content was 3.7 ± 0.9 mg collagen/construct in 76 rpm as compared to 2.5 ± 0.5 mg collagen/construct (Figure 11C). On day 24, the construct collagen content was similar between 76 rpm and DHL at 2.1 ± 0.5 mg collagen/construct and 2.3 ± 0.6 mg collagen/construct respectively. After 35 days in bioreactor culture, the construct collagen content was lower in DHL as compared to 76 rpm, the values are 1.9 ± 0.6 mg collagen/construct and 3.7 ± 0.6 mg collagen/construct, respectively (NS $p = 0.08$).

Thus, decreasing hydrodynamic loading increases construct GAG content by ~40% after 35 days in bioreactor culture as compared to steady loading at 76 rpm.

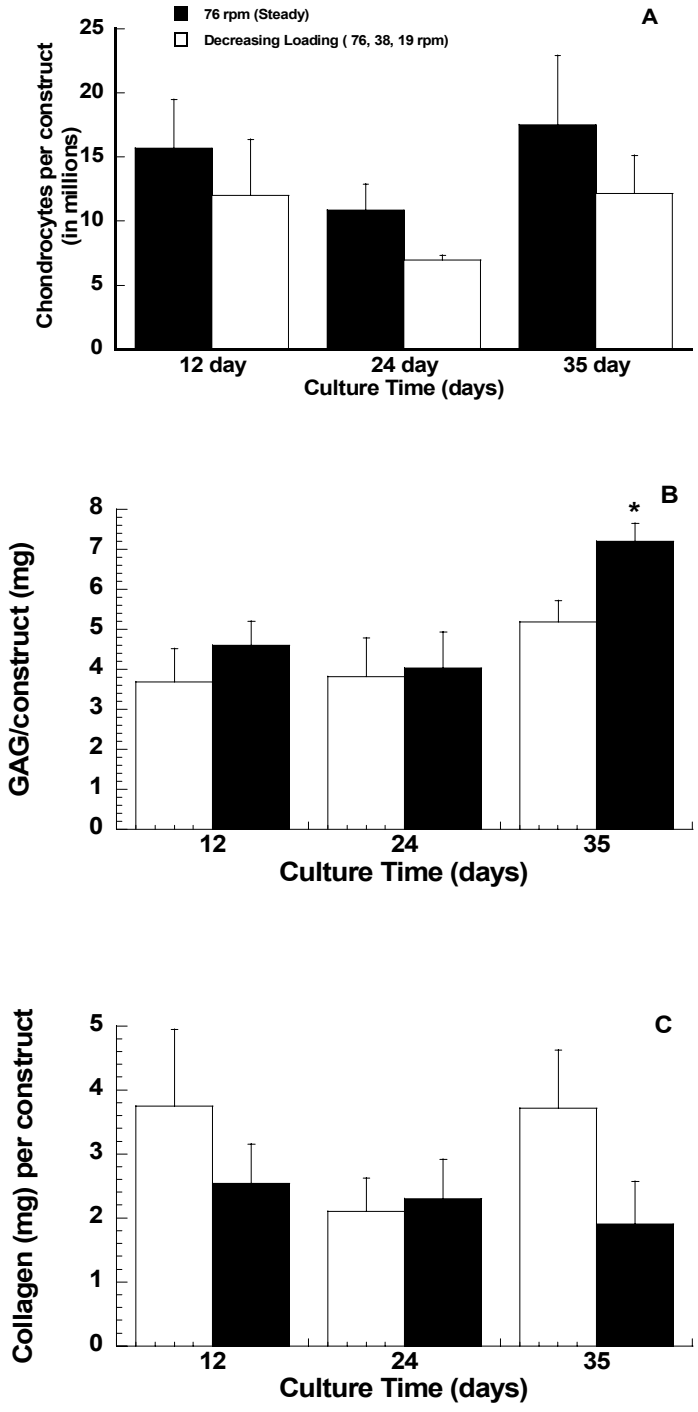


Figure 11: Effect of DHL on construct development. Data are mean ± SEM for N=4 experiments, n=10-16 constructs (A) Chondrocytes per construct (in millions), (B) GAG per construct (mg), (C) Collagen per construct (mg). The construct GAG content is significantly higher on day 35 for DHL as compared to 76 rpm (*$p<0.05$)

Effect of increasing hydrodynamic loading on construct development

The primary objective of this study was to determine the effect of increasing hydrodynamic loading on construct development. The increasing hydrodynamic loading regimen follows the opposite trend of the decreasing hydrodynamic loading regimen. The idea behind this loading regimen was to disseminate the effect of varying shear stress on construct surface over the culture period. This study was designed after the decreasing hydrodynamic loading was carried out. The main objective of this study was to see if increasing hydrodynamic loading would further enhance construct extracellular matrix deposition.

As in most of the bioreactor experiments, cell proliferation is robust in either bioreactor condition (Figure 12A). After 12 days in the bioreactor, the chondrocyte content per construct was 3.1 ± 0.6 million in the bioreactor operated at 19 rpm and 5.8 ± 0.5 million in the bioreactor operated under the increasing hydrodynamic loading regimen. The cell content was similar on days 24 and 35. In the 19 rpm bioreactor, the cell content was 9.4 ± 1.44 and 16.1 ± 1.9 million chondrocytes per construct on days 24 and 35 respectively. In the increasing hydrodynamic loading regimen, the cell content was 6.3 ± 0.7 and 11.1 ± 0.9 million chondrocytes per construct on days 24 and 35 respectively.

Construct collagen content was similar on day 12 and 24 (Figure 12B). In the 19 rpm bioreactor, construct collagen content was 1.2 ± 0.1 and 1.0 ± 0.1 mg per construct on days 12 and 24 respectively whereas in the IHL bioreactor, the construct collagen content was 1.1 ± 0.1 and 1.3 ± 0.07 mg per construct on

days 12 and 24 respectively. On day 35, the construct collagen content was slightly higher in the IHL bioreactor. The construct collagen content was 1.6± 0.2 and 2 ± 0.3 mg per construct in the 19 rpm and the IHL bioreactor respectively (NS $p<0.1$).

The construct GAG content showed similar trends for days 12 and 24 in the 19 rpm and the IHL bioreactor (Figure 12C). In the 19 rpm bioreactor, construct GAG content was 2.3 ± 0.3 and 3.9 ± 0.5 mg per construct on days 12 and 24 respectively. In the IHL bioreactor, construct GAG content was 2.8 ± 0.5 and 2.1 ± 0.4 mg per construct on days 12 and 24 respectively. On day 35, the 19 rpm had higher GAG content at 7.8 ± 1.6 mg per construct and in the IHL bioreactor 5.3 ± 0.5 mg per construct (NS $p<0.1$).

The increasing hydrodynamic loading regimen does not show an increase in construct GAG and collagen as compared to the steady loading of 19 rpm. The construct GAG content was higher in the 19 rpm bioreactor indicating that the increasing hydrodynamic loading was not as favorable to construct extracellular matrix as compared to decreasing hydrodynamic loading.

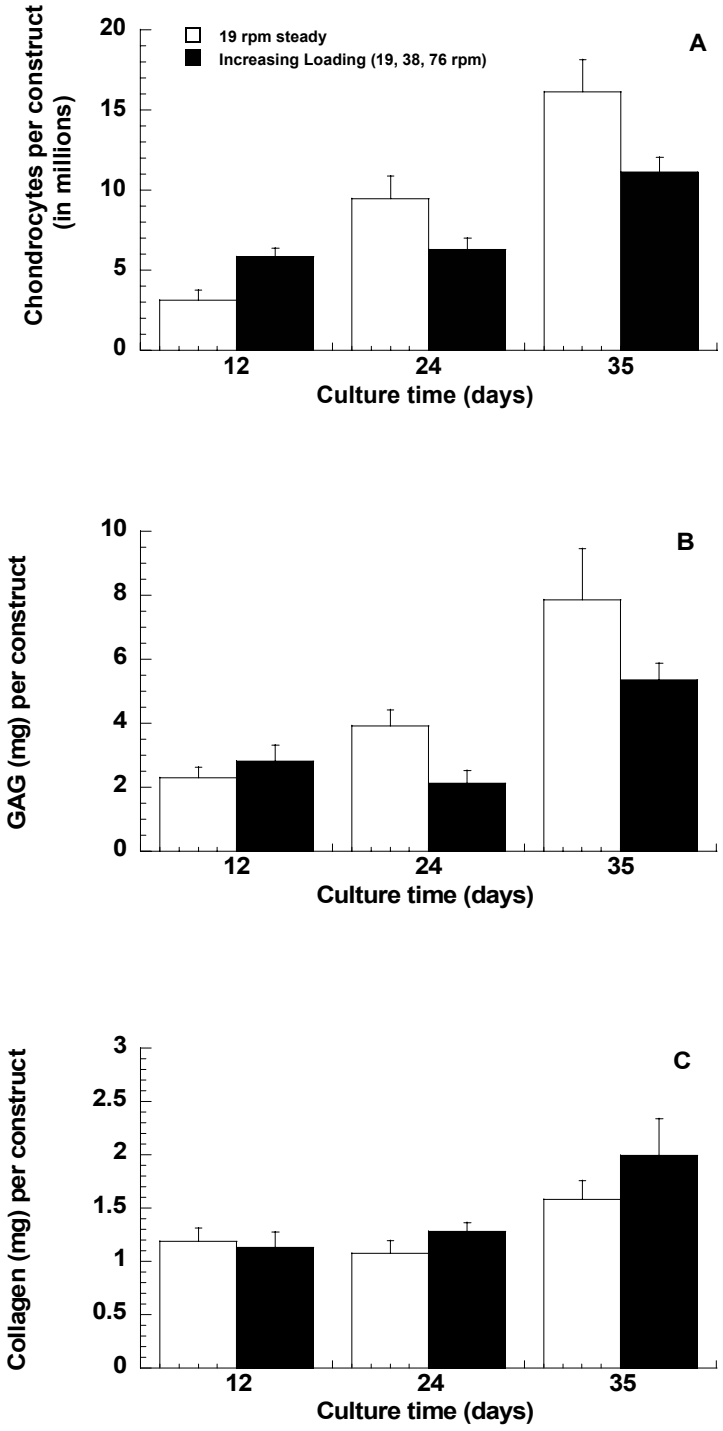


Figure 12: Effect of IHL on construct development. Data are mean ± SEM from N=3 experiments, n=14-19 constructs (A) Chondrocytes per construct (in millions), (B) GAG per construct (mg), (C) Collagen per construct (mg). No statistically significant differences were found between IHL and 19 rpm.

Histological Analyses

Histological analyses of the constructs under the four loading conditions are presented here. Routine hematoxylin and eosin stains showed the presence of cells (Figure 13). Residual polymer (marked by arrows) was observed as transclucent material in the sections. Very little residual polymer was seen in most sections. The 76 rpm construct had a 'holey' appearance and it was not immediately apparent whether this was due to a processing artifact or due to the nature of the construct.

Masson's trichrome stain for collagen (Figure 14) showed the presence of collagen in all the samples. The collagen fibers stain blue in the construct. The staining in the 76 rpm construct was most intense despite the appearance of the construct. However, the fibers did not seem aligned but rather 'bunched up' in this construct as compared to the other sections.

Safranin-O staining for GAG showed the presence of GAG in all the sections (Figure 15). The GAGs are stained orange-red in the constructs. The decreasing hydrodynamic loading had the most intense stain for GAG. Very little GAG was seen in the 76 rpm constructs. The 19 rpm and the increasing loading constructs were characterized by large parts of the section where no stain for GAG was observed.

Immunofluorescence for collagen type II (Figure 16) showed the presence of type II collagen in all the constructs. The decreasing loading construct had the most uniform distribution of the type II collagen while 76 rpm had the most random distribution. Less intense staining was seen for 19 rpm and increasing loading.

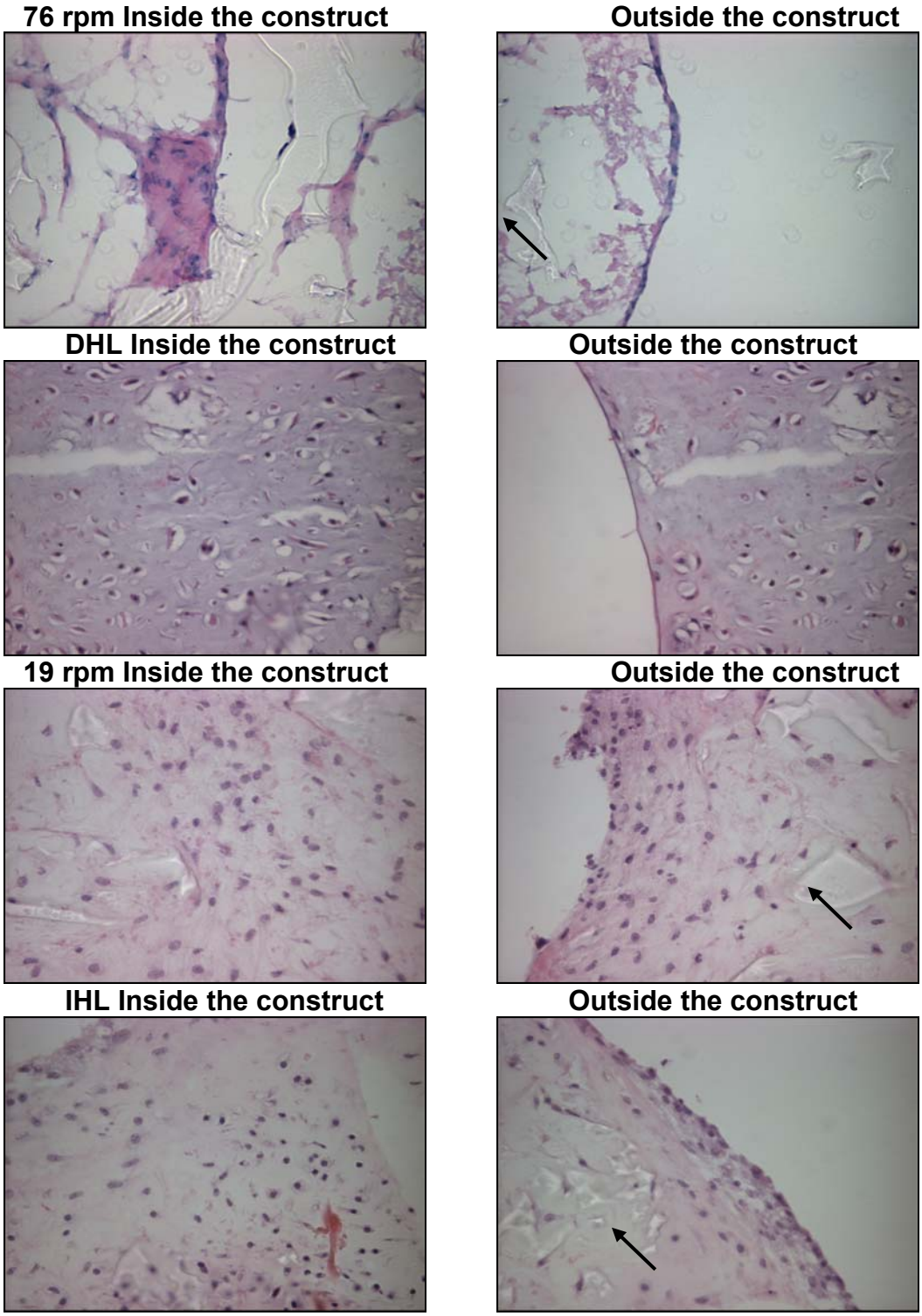


Figure 13: H & E stain for constructs. Images are all taken at 20X with the same light intensity for all the sections. The cell nuclei are stained dark purple and the extracellular matrix is light pink in color. Remnants of the polymer scaffold are seen as translucent parts, marked by arrows.

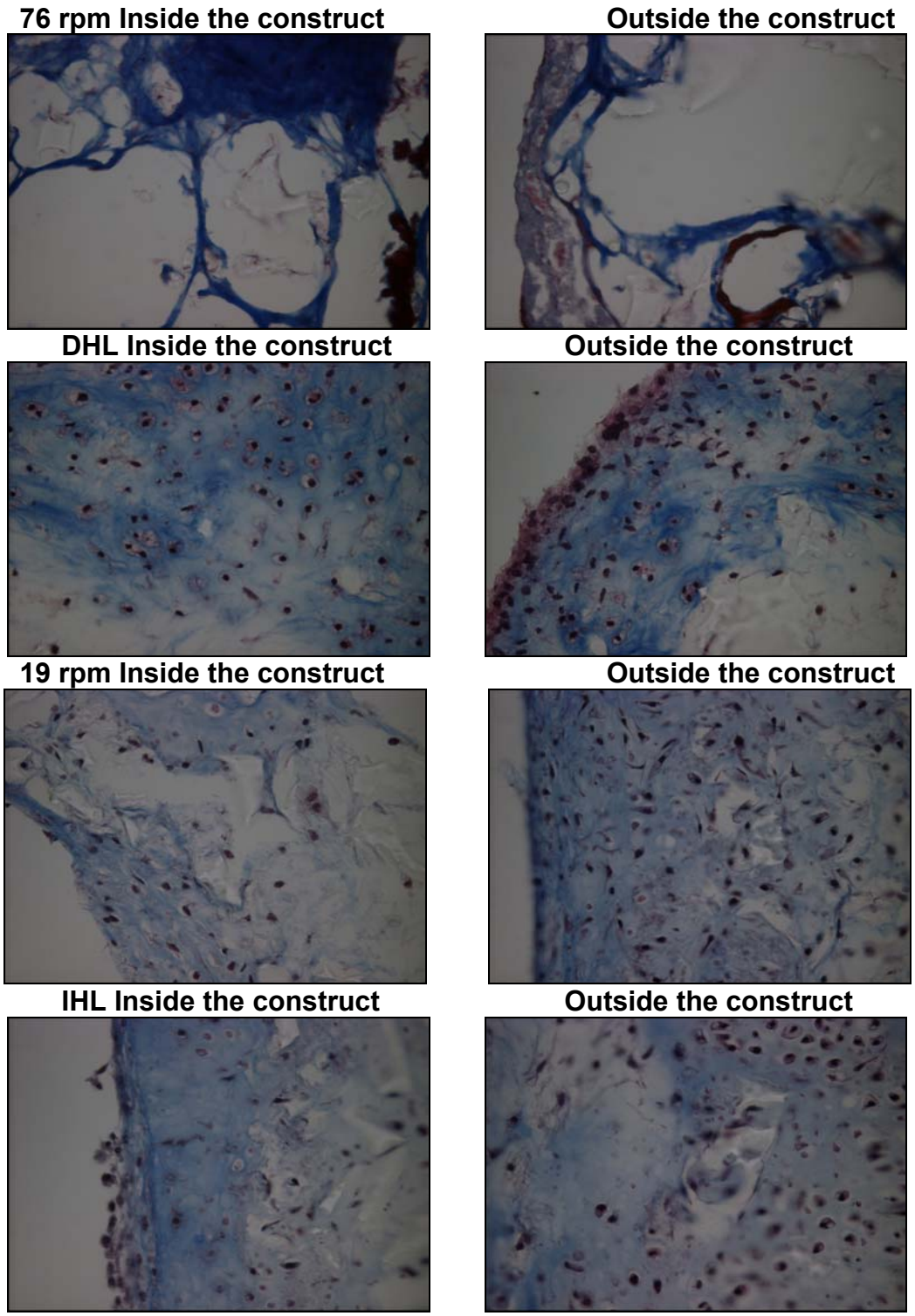


Figure 14: Masson's trichrome stain for collagen. Images are taken at the same light intensity for all sections. The cell nuclei are stained dark purple and the collagen in the construct is stained blue.

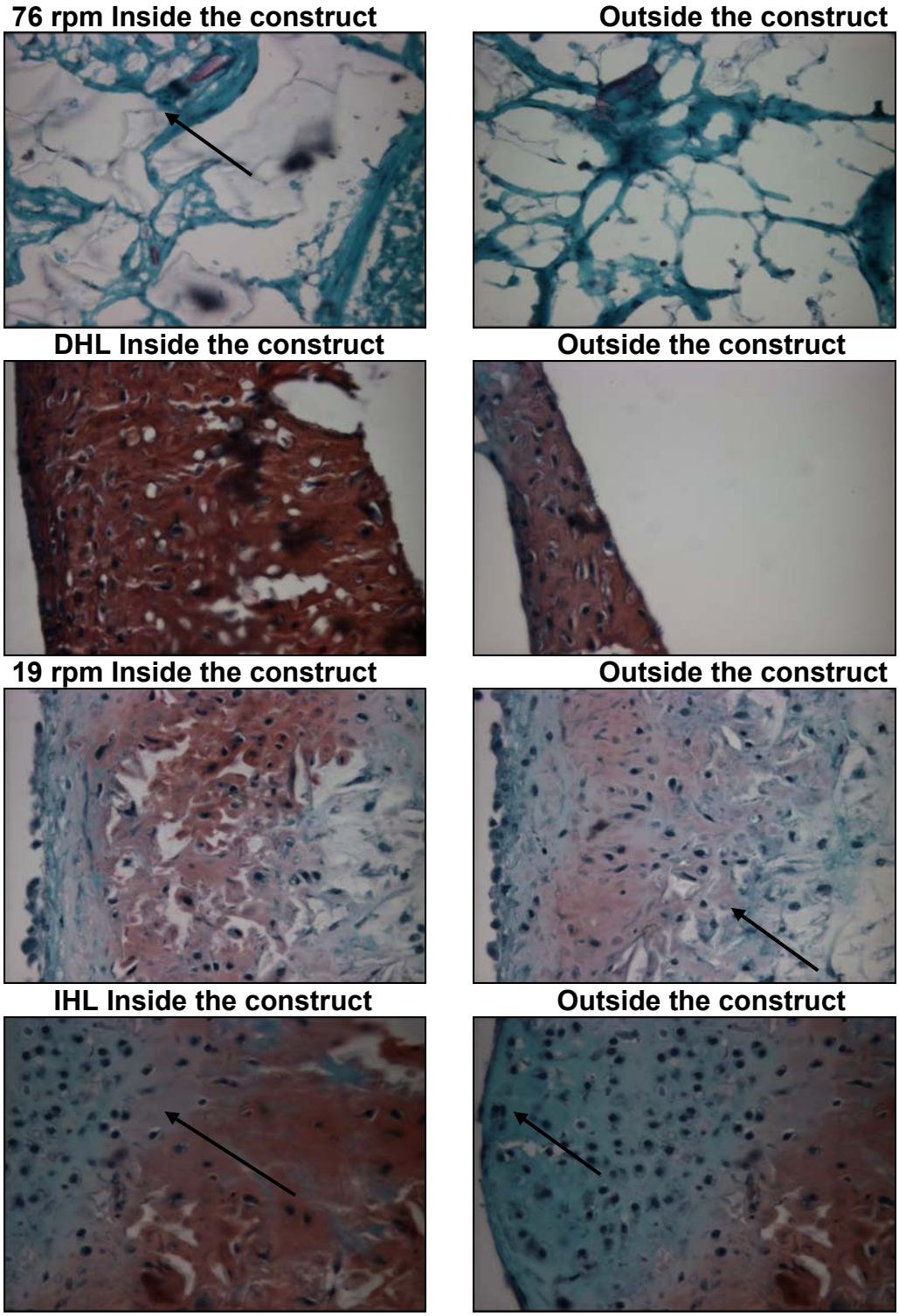


Figure 15: Safranin-O stain for GAG. The images are taken at 20X magnification at the same light intensity for all sections. The cells in the sections are stained black and the GAG is stained orange-red. In the 19 rpm, IHL and 76 rpm sections, there are areas of the construct that do not stain for GAG, marked by arrows.

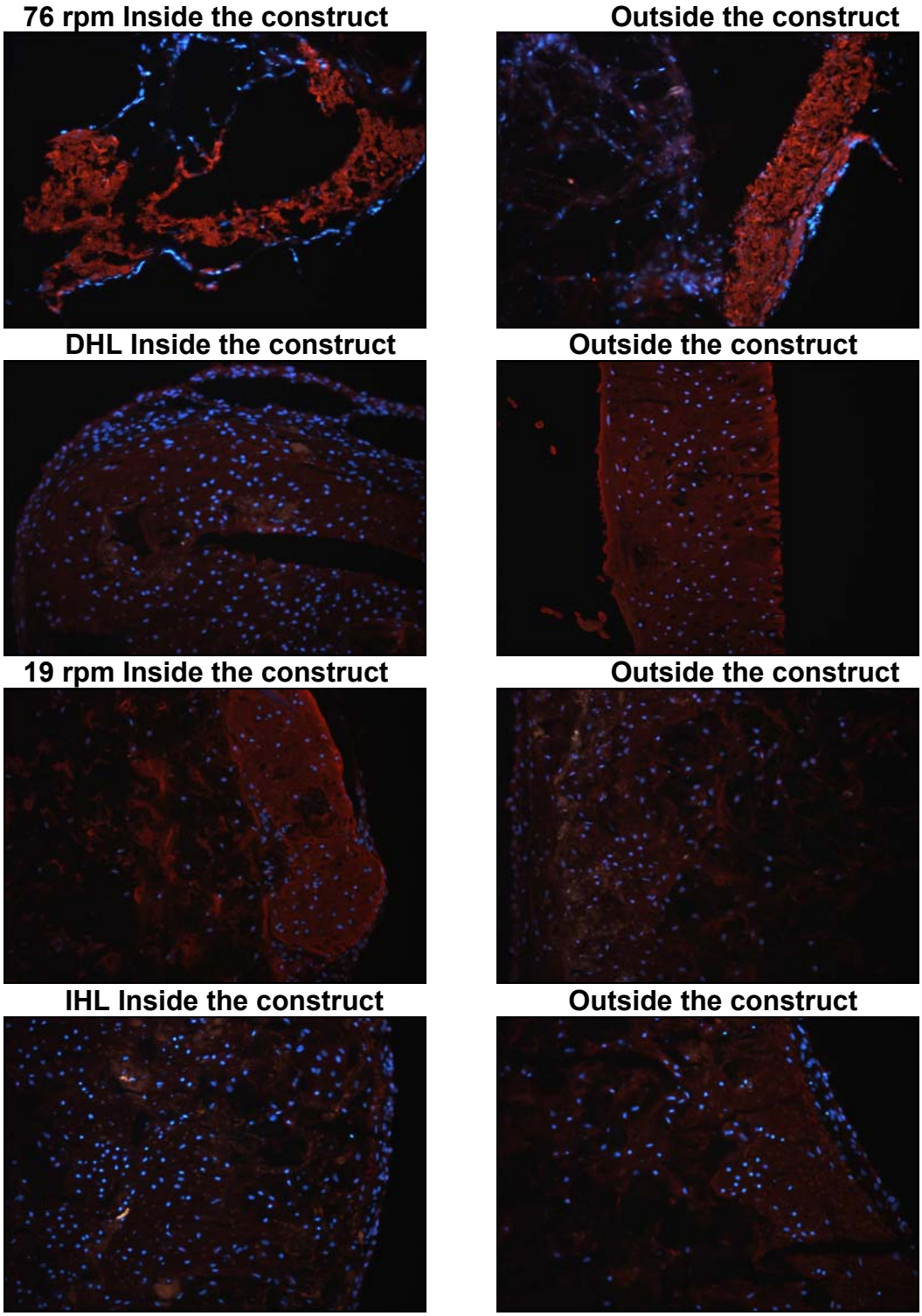


Figure 16: Immunofluorescence for type II collagen. The images were taken using a fluorescent microscope. The cell nuclei are stained blue using the Hoechst dye and the collagen II content is stained red using the Texas Red fluorophore

Effect of combining decreasing hydrodynamic loading with low oxygen tension

In the two different hydrodynamic loading regimens compared in this study, the decreasing hydrodynamic loading regimen had the most favorable effect on construct GAG deposition. Overall, decreasing loading increased matrix GAG accumulation whereas increasing hydrodynamic loading decreased accumulation of GAG in the construct matrix, compared to their respective steady hydrodynamic loading controls. In contrast, hydrodynamic loading conditions only marginally affected collagen accumulation. Therefore, the low oxygen studies were carried out in the bioreactor with decreasing hydrodynamic loading regimen as the mechanical stimulation. Based on the results of the DHL regimen, and the low oxygen tension studies, these two stimuli were combined in an attempt to increase construct collagen content.

Chondrocyte proliferation was robust in both 20% oxygen tension and 5% oxygen tension cultures. The initial cell seeding number on day 0 was 6.25 million chondrocytes per construct in either oxygen tension. After 35 days in bioreactor culture, cell proliferation was similar at both oxygen tensions with cell numbers being 38 ± 9 and 24 ± 6 million chondrocytes per construct for 5% and 20% oxygen tension culture conditions (Figure 17A). The chondrocyte number per construct was 57 % higher in low oxygen tension after 35 days culture as compared to normoxia, although this difference is statistically not significant.

Construct GAG composition (Figure 17B) was 13.4 ± 3.2 mg per construct in low oxygen tension bioreactor culture and higher than that observed in the 20% oxygen tension culture (5.5 ±1.6 mg GAG per construct) after 12 days.

Construct GAG levels increased during culture for both oxygen tension conditions between 12 and 24 days. After 35 days, culture under low oxygen tension led to an overall 40% increase in GAG composition in constructs compared to culture in 20% oxygen tension (15.8 ± 3.3 DHL, 11.6 ± 3.4 mg GAG per construct, respectively). However, the difference in GAG composition was not significantly different in constructs cultured under 5% or 20% oxygen tension at any time point.

Construct collagen content was higher at all time points in low oxygen tension bioreactor culture as compared to normoxic culture (Figure 17C). After 24 days culture, constructs cultured at 5% oxygen tension contained 8.0 ± 1.8 mg collagen per construct as compared to 1.8±0.4 mg collagen per construct (*$p < 0.05$) for constructs cultured in 20% oxygen tension. After 35 days culture, 5% oxygen tension constructs had 6.5±1.4 mg collagen/construct as compared to 2.1±0.5 mg collagen/construct in the 20% oxygen tension cultures (*$p < 0.05$).

Indirect ELISA for collagen type II content in the constructs showed trends similar to that of total collagen content in the construct. Greater than 70% of the total collagen content in the construct was type II collagen. After 35 days in bioreactor culture, the type II collagen content was 6.3±1.8 mg/construct in hypoxia and 2.4±1.0 mg/construct in normoxia (Figure 18A). The type II collagen content was higher for constructs cultured under 5% oxygen tension as compared to constructs cultured under 20% oxygen tension at all time points. Type I collagen ELISA showed that less than 0.5 mg was collagen I was present in the constructs at all time points in either oxygen tension (Figure 18B).

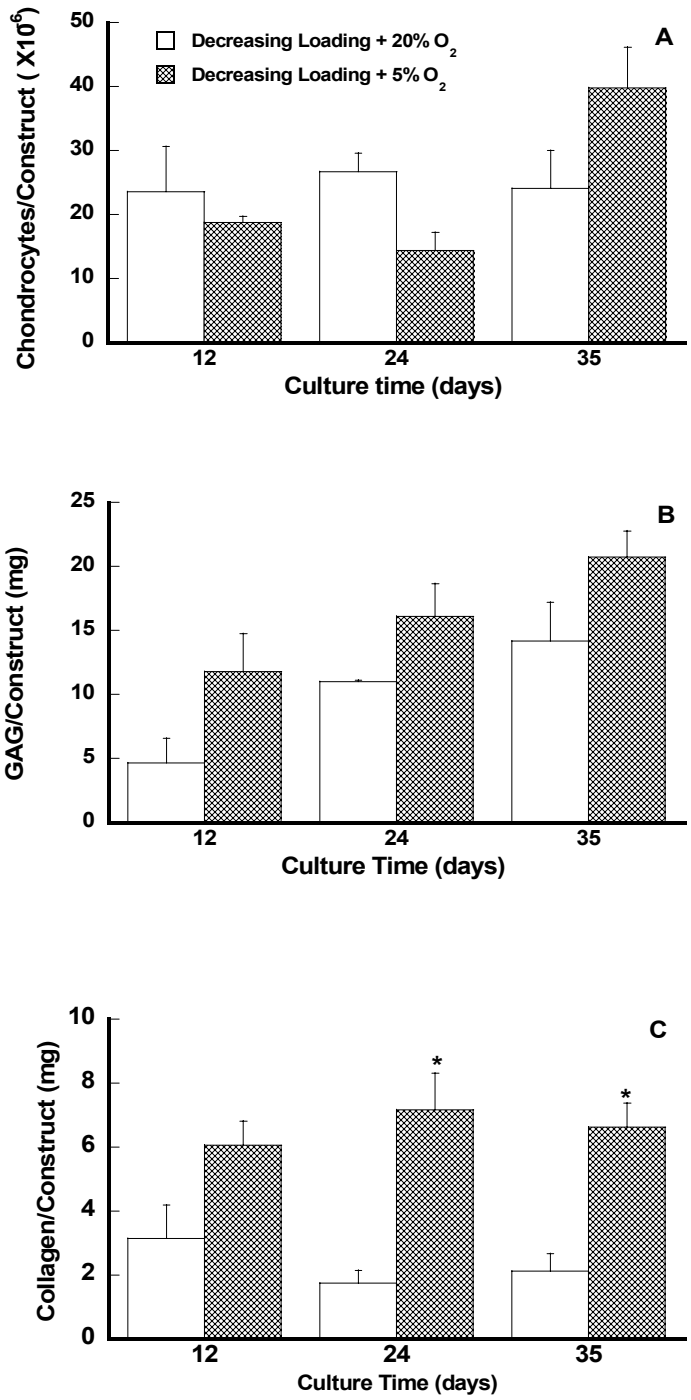


Figure 17: Construct composition under low oxygen tension with decreased hydrodynamic loading. Data are mean ± SEM from N=3 experiments for 20% oxygen tension + DHL and N=4 experiments for 5% oxygen tension + DHL. (n=12-20 constructs) (A) Chondrocytes per construct (in millions, (B) GAG per construct (mg), (C) Collagen per construct (mg). * $p < 0.05$

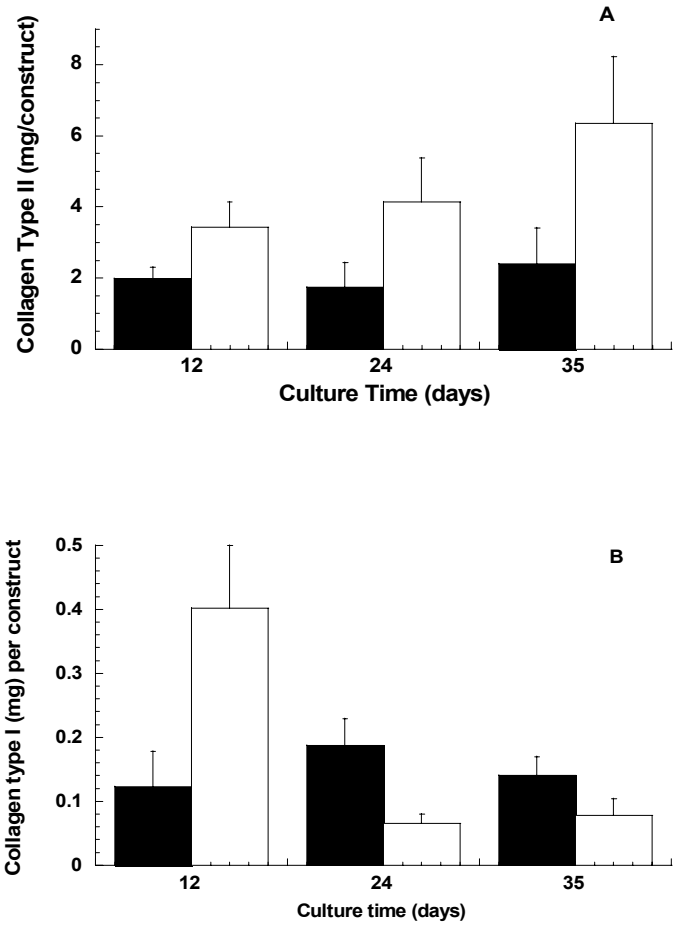


Figure 18: Construct collagen characterization for low oxygen tension in the presence of decreasing hydrodynamic loading. Data are mean ± SEM from N=3 experiments for 20% oxygen tension + DHL and N=4 experiments for 5% oxygen tension + DHL. (A) Collagen type II per construct (mg), (B) Collagen type I per construct (mg)

Material properties

The dynamic shear modulus of constructs cultured in the presence of both low oxygen tension and decreasing hydrodynamic loading was measured to determine the stiffness of the construct.

The dynamic shear modulus for constructs cultured under 5% oxygen tension with decreasing hydrodynamic loading is higher at all frequencies of measurement compared to 20% oxygen tension culture with decreasing hydrodynamic loading (Figure 19A). Constructs cultured in 20% oxygen tension have shear moduli of 131 ± 26 kPa at 1Hz, 67 ± 23 kPa at 0.1 Hz and 64 ± 20 kPa at 0.01Hz. The constructs cultured in hypoxia have shear moduli of 345 ± 213 kPa at 1 Hz, 285 ± 171 kPa at 0.1Hz and 247 ± 152 kPa at 0.01Hz. At all frequencies of testing, the shear modulus at 5% was higher than at 20% oxygen tension. The values of shear moduli are significantly higher at 5% oxygen tension than 20% oxygen tension at 0.01 Hz and 0.1 Hz ($*p < 0.05$). The increase in the dynamic shear moduli of the constructs with frequency indicates that the construct rheological properties are frequency dependent. The phase angle or loss angle (δ) is shown in Figure 19B.

The dynamic shear modulus of PLLA scaffolds soaked in complete culture medium for 35 days either under 5% or 20% oxygen tension was measured to compare the behavior of polymer alone versus constructs containing extracellular matrix components. The shear moduli of the scaffolds were in the same range as that of the constructs (Figure 20). However, while the constructs remained intact after the testing process, the polymer scaffolds exhibited cracks on the

surface and disintegrated into fine powder at the end of the testing process indicating that the scaffolds are fragile. The frequency dependence of the shear, viscous and elastic modulus is different for the scaffold than from the construct and the sudden increase in the moduli between 0.1 Hz and 1.0 Hz indicates that the scaffold is brittle and cannot withstand high impact loading or testing.

Combining low oxygen tension with decreasing hydrodynamic loading increased construct collagen content 3-fold and GAG content by ~40%. The dynamic shear modulus for the constructs was higher for the constructs cultured under low oxygen tension with decreasing hydrodynamic loading by ~3-4 fold. This indicated that the combination of these conditions is favorable for increasing construct extracellular matrix production.

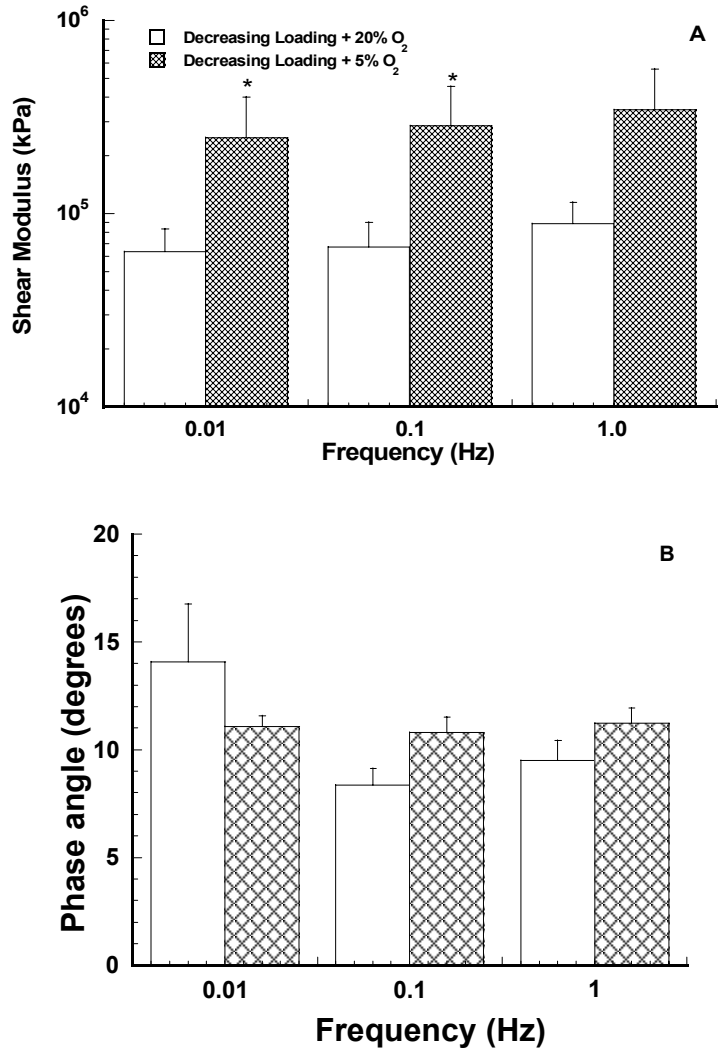


Figure 19: Construct dynamic shear modulus and phase angle for constructs cultured under low oxygen tension and decreasing hydrodynamic loading. Data are mean ± SEM from N=3 experiments for 20% oxygen tension and N=4 for 5% oxygen tension (A) Construct shear modulus (kPa), (B) phase angle (degrees).

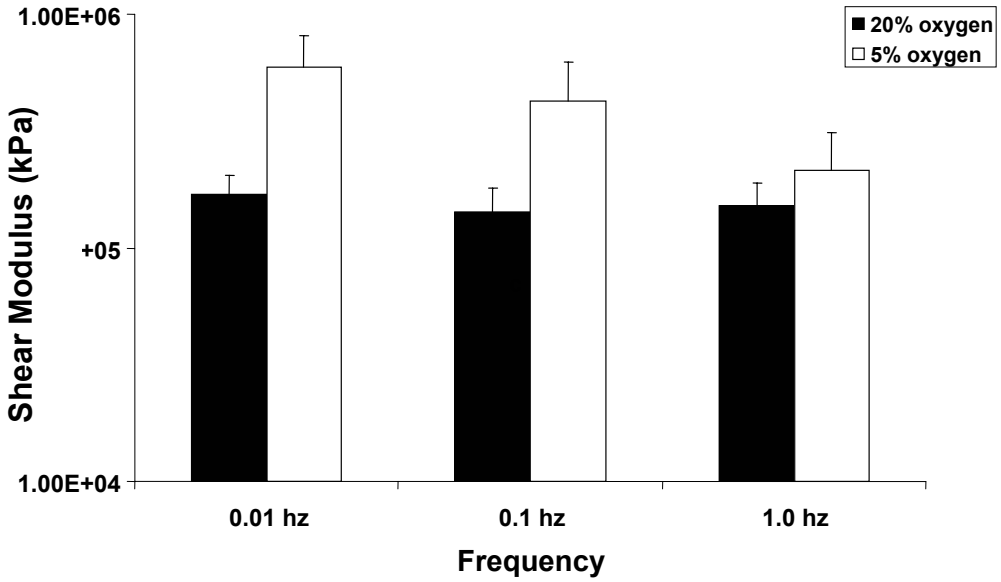


Figure 20: Shear modulus of unseeded PLLA scaffolds soaked in complete DMEM for 35 days under 20% and 5% oxygen tension. Data are mean ± SEM for N=3 constructs.

Histological analyses

Comparing the DHL + 5% oxygen and DHL + 20% oxygen, constructs from either culture condition show uniform chondrocyte distribution through the construct thickness. Chondrocytes cultured in a 5% oxygen tension have a more rounded morphology as compared to chondrocytes cultured under 20% oxygen (Figure 21). Staining for collagen and GAG is more intense in the low oxygen tension as compared to normoxic constructs when observed under light microscopy, consistent with higher matrix accumulation under hypoxia as shown in Figures 17 and 18. Type II collagen was found in both the normoxic and hypoxic culture constructs indicating that the nature of the construct was cartilage-like.

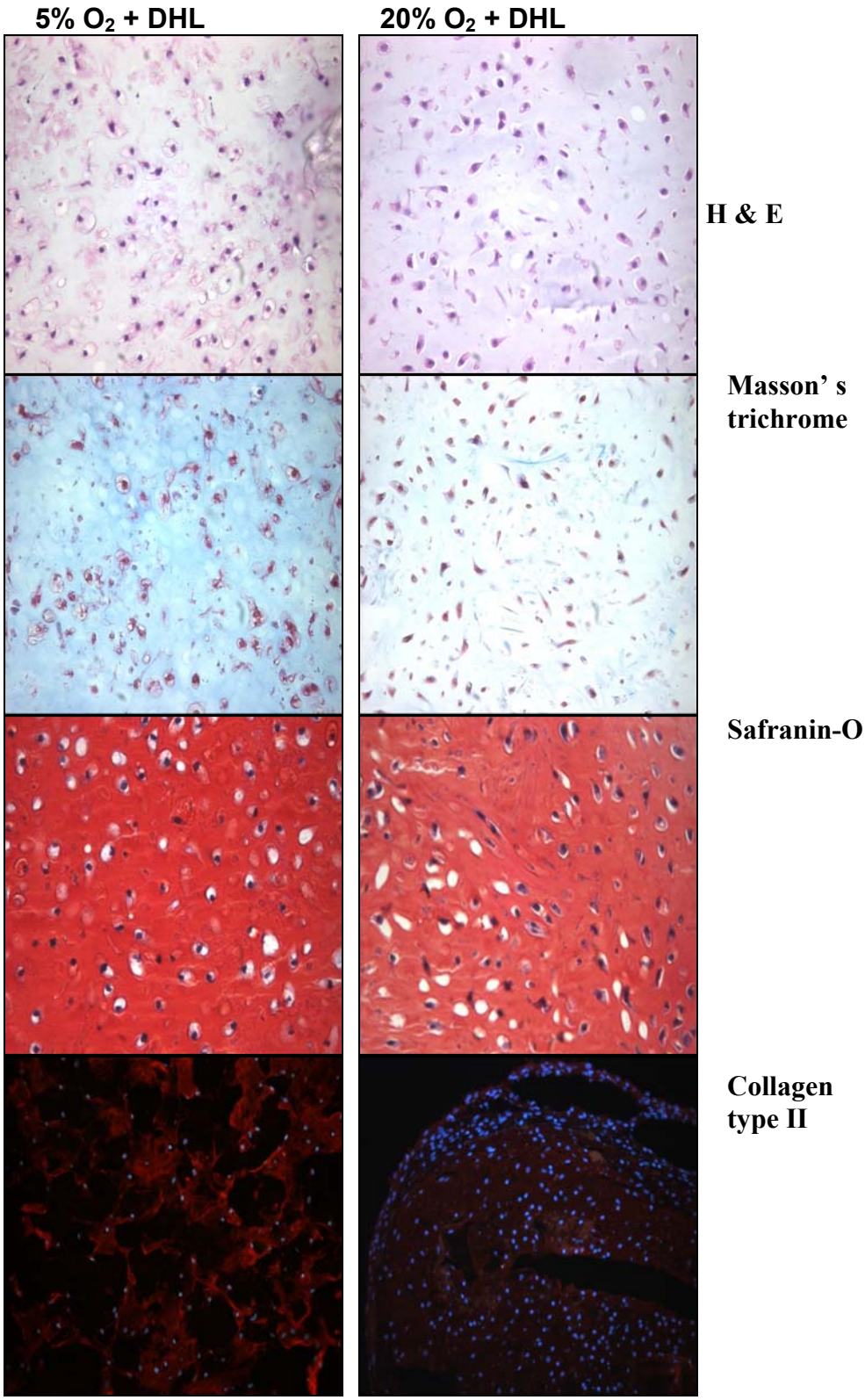


Figure 21: Histology images for constructs cultured under low oxygen tension and DHL. Shown are representative stains for cells, GAG, collagen and type II collagen.

Medium analysis

The conditioned medium from the bioreactor was saved for biochemical analyses every time medium was changed. Table 9 shows the NO production in different bioreactor conditions measured by nitrate/nitrite accumulation. On day 12, the NO production was lowest in the 19 rpm and IHL bioreactors. In the DHL bioreactor, as the shear stress decreased over culture period, the NO production changed, with the production being lowest between days 24 and 35. There were no trends or significant differences observed in the DHL between 5% and 20% oxygen over the culture period.

Table 9: NO production in the different bioreactor conditions.

Bioreactor	12 day	24 day	35 day
76 rpm	0.09 ± 0.02	0.111 ± 0.018	0.08 ± 0.02
DHL	0.12 ± 0.01	0.153 ± 0.038	0.09 ± 0.03
19 rpm	0.06 ± 0.03	0.03 ± 0.008	0.02 ± 0.005
IHL	0.06 ± 0.04	0.07 ± 0.02	0.03 ± 0.01
DHL 20% oxygen	0.14 ± 0.08	0.10 ± 0.04	0.10 ± 0.03
DHL 5% oxygen	0.18 ± 0.09	0.06 ± 0.018	0.08 ± 0.02

Effect of TGF-β1 at steady shear stress

TGF- β1 is a known modulator of chondrocyte response in vitro. To assess the effect of TGF- β1 in bioreactor culture, two bioreactor conditions were compared. The control bioreactor was operated at 38 rpm with complete DMEM. The test bioreactor was operated at 38 rpm with complete DMEM and 0.2 ng/ml of TGF-β1. The experiment was run for 24 days.

Chondrocyte proliferation in the presence of TGF-β1 is much higher compared to chondrocyte proliferation in the control bioreactor (Figure 22A). After 12 days in bioreactor culture, the TGF- β bioreactor had 65 million cells per construct and the control bioreactor had 8.2 million chondrocytes. Between 12 and 24 days, the cell population in the presence of TGF- β1 decreased to almost half. The construct collagen content was similar in either culture condition, on day 12 either bioreactor yielded constructs with approximately 0.7 mg collagen and on day 24, about 2.2 mg (Figure 22C). However, the construct GAG content was higher on days 12 and 24 in the presence of TGF-β1 almost twice as much as in the control bioreactor. The constructs in the control bioreactor had 4.5 mg and 5.3 mg GAG per construct on days 12 and 24 respectively. The constructs cultured in the presence of TGF-β1 had 8.4 and 9.2 mg GAG per construct on days 12 and 24 respectively (Figure 22B). This preliminary experiment indicated that the presence of TGF-β1 was beneficial to chondrocyte proliferation early in culture and stimulated GAG production.

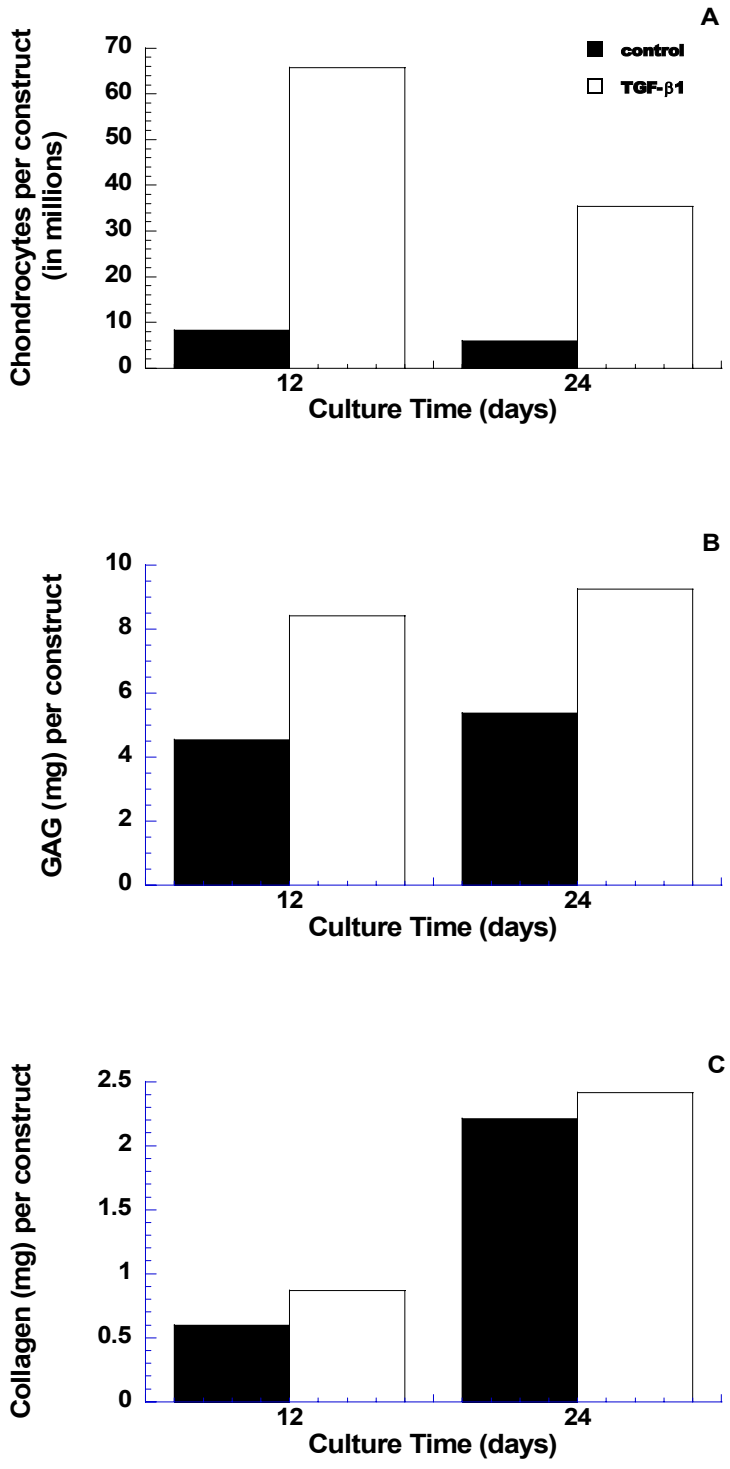


Figure 22: Effect of TGF- β1 on construct development. Data are from one experiment. (A) Chondrocytes per construct (in millions), (B) GAG per construct (mg), (C) Collagen per construct (mg)

Effect of TGF-β1 in the presence of decreasing hydrodynamic loading

Among the different hydrodynamic loading regimens studied, the decreasing hydrodynamic loading has the most beneficial effect on construct GAG accumulation in normoxia. In order to determine if the combined presence of TGF-β1 and decreasing hydrodynamic loading would enhance construct extracellular matrix further, experiments were carried out to determine the effect of 0.2 ng/ml of TGF-β1 in the decreasing loading regimen.

The constructs cultured in the presence of TGF-β1 and DHL contained 32.1 ± 12.2 million cells/construct (Figure 23A) on day 12. On day 24, the chondrocyte population in the construct decreased to 8.3 ± 1.6 million chondrocytes/construct. On day 35, the construct cell number is 10.2 ± 1.5 million chondrocytes/construct.

The construct GAG content was 4.1 ± 0.7 mg GAG/construct (Figure 23B) on day 12. On day 24, the construct GAG content was 4.3 ± 0.6 mg GAG/construct. At the end of culture on day 35, the construct GAG content was 4.5 ± 0.5 mg GAG/construct.

On day 12, the construct collagen content was 3.0 ± 0.9 mg collagen/construct (Figure 23C). On day 24, the construct collagen content was 3.3 ± 0.9 mg collagen/construct. After 35 days in bioreactor culture, the construct collagen content was 1.7 ± 0.5 mg collagen/construct (Figure 23C).

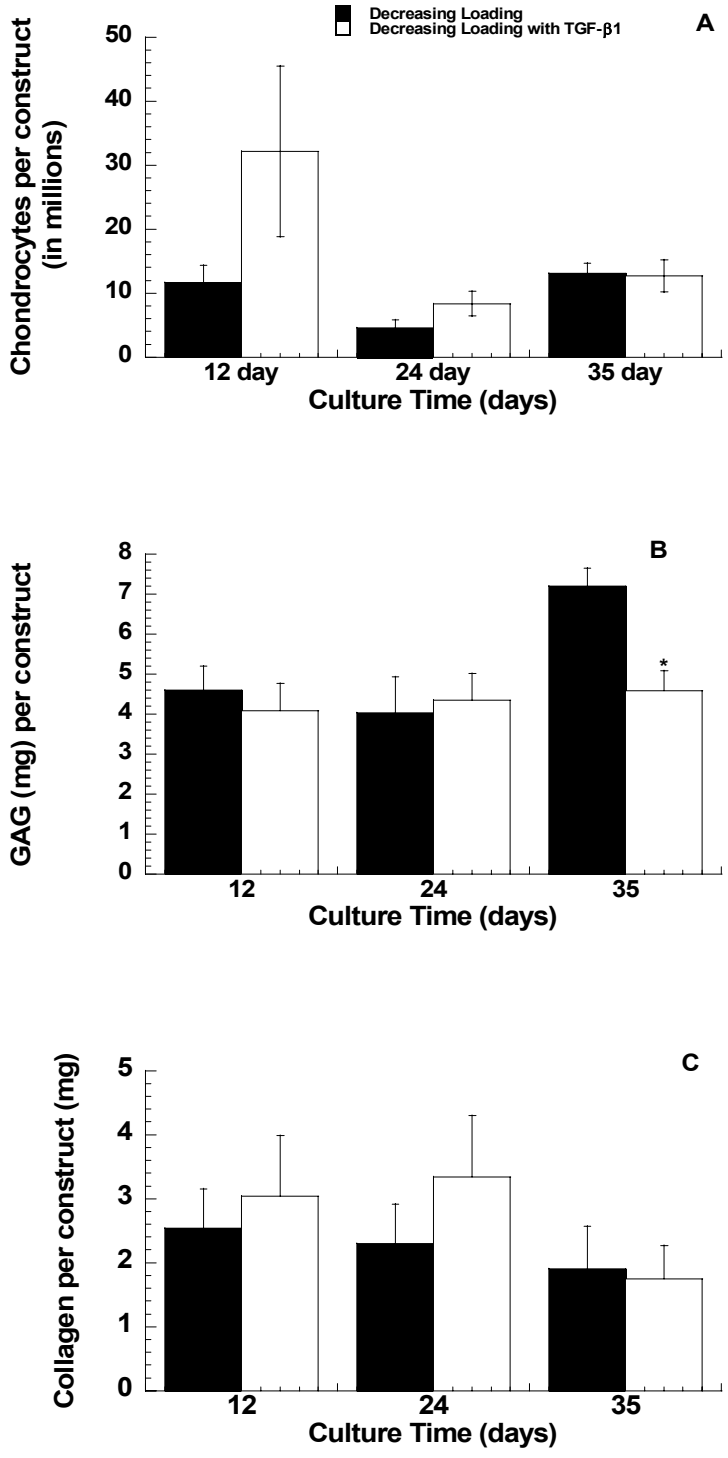


Figure 23: Effect of TGF- β1 with DHL. Data are mean ± SEM from N=4 experiments. (A) Chondrocytes per construct (in millions), (B) GAG per construct (mg), (C) Collagen per construct (mg)

Perfusion shear flow bioreactor

The perfusion bioreactor was designed to incorporate the hydrodynamic loading features of the concentric cylinder bioreactor along with perfusion flow to enhance the ultrastructure of tissue engineered cartilage. This bioreactor was characterized for some of the basic functionalities of tissue engineering bioreactors. The first study carried out in the bioreactor was to determine seeding efficiency and cell viability in the constructs early in culture. Four constructs each were harvested on days 2, 3 and 4 post-seeding. One construct each was used for confocal imaging of live/dead cells and histology. The construct cell content was determined by the DNA assay. The initial cell seeding was 150 million cells per 16 constructs which is about 9.375 million chondrocytes per construct. After 2 days, only 1 million chondrocytes per construct had attached. This amounted to a cell seeding efficiency of about 10%. This could be because of the large volume of culture medium held in the outer cup (~ 500 ml). The chondrocyte content over the seeding period is shown in figure 24. The chondrocyte content decreased from 1 million to about 0.5 million chondrocytes per construct. This could be because of the cells not completely attached or being released in to the media because of the large porosity of the construct. The cell viability was high as is shown in Figure 25. This showed that although the cell number decreases over the 4 day period, there is no evidence of cell death. Decrease in cell number could also be because of potential nutrient gradient inside the construct due to lack of perfusion flow.

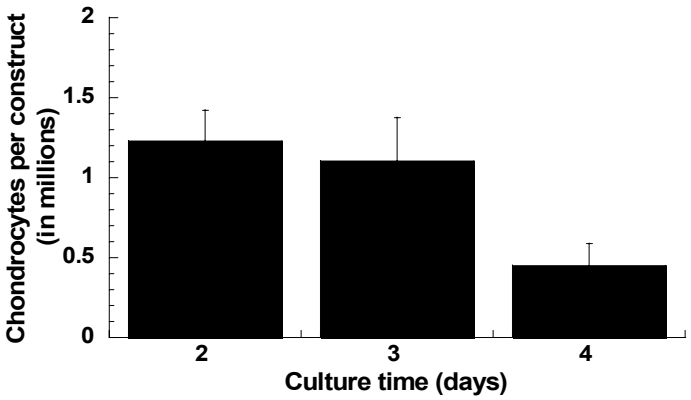


Figure 24: Construct seeding in the perfusion bioreactor. Construct cell numbers are shown as mean ± SEM from N=4 experiments.

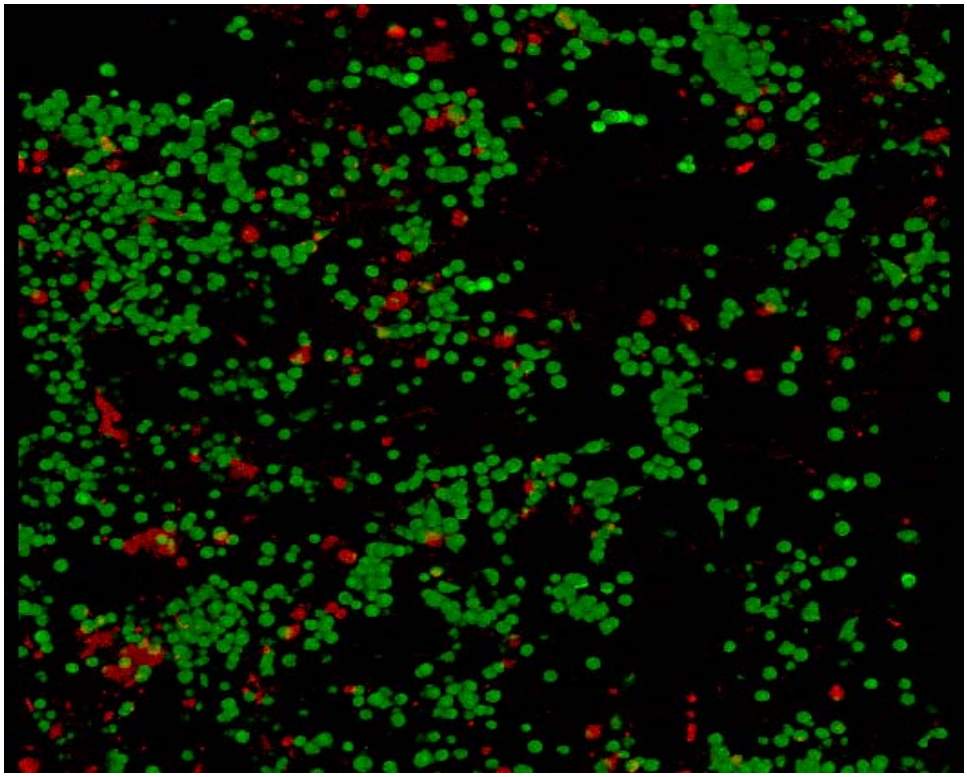


Figure 25: Chondrocyte viability: Live/dead assay showing chondrocyte viability after 4 days in the perfusion bioreactor

Short term construct development

Short term construct development studies in the perfusion shear flow bioreactor were carried out for two weeks. The seeding phase was carried out similar to the four day experiments with no perfusion flow. Perfusion flow was from day 4 to day 14. Four constructs were harvested at each time point.

The construct cell proliferation showed an increase over time (Figure 26A). The chondrocyte number per construct increased from 1.2 ± 0.2 million per construct on day 4 to 3.3 ± 0.4 million per construct on day 14. The construct cell number on day 14 was significantly higher than the construct cell number per construct on days 4 and 7. This indicates that the presence of perfusion and shear is favorable for chondrocyte proliferation in the bioreactor. The construct collagen content was 0.8 ± 0.14 mg per construct on day 4 and on day 14 it was 1.2 ± 0.14 mg per construct (Figure 26B). There was no significant change in the construct collagen content over the short culture period. The construct GAG content showed an exponential increase over the culture period (Figure 26C). The construct GAG content increased from 3±0.4 mg per construct on day 4 to 10.3 ± 2.6 mg per construct on day 14.

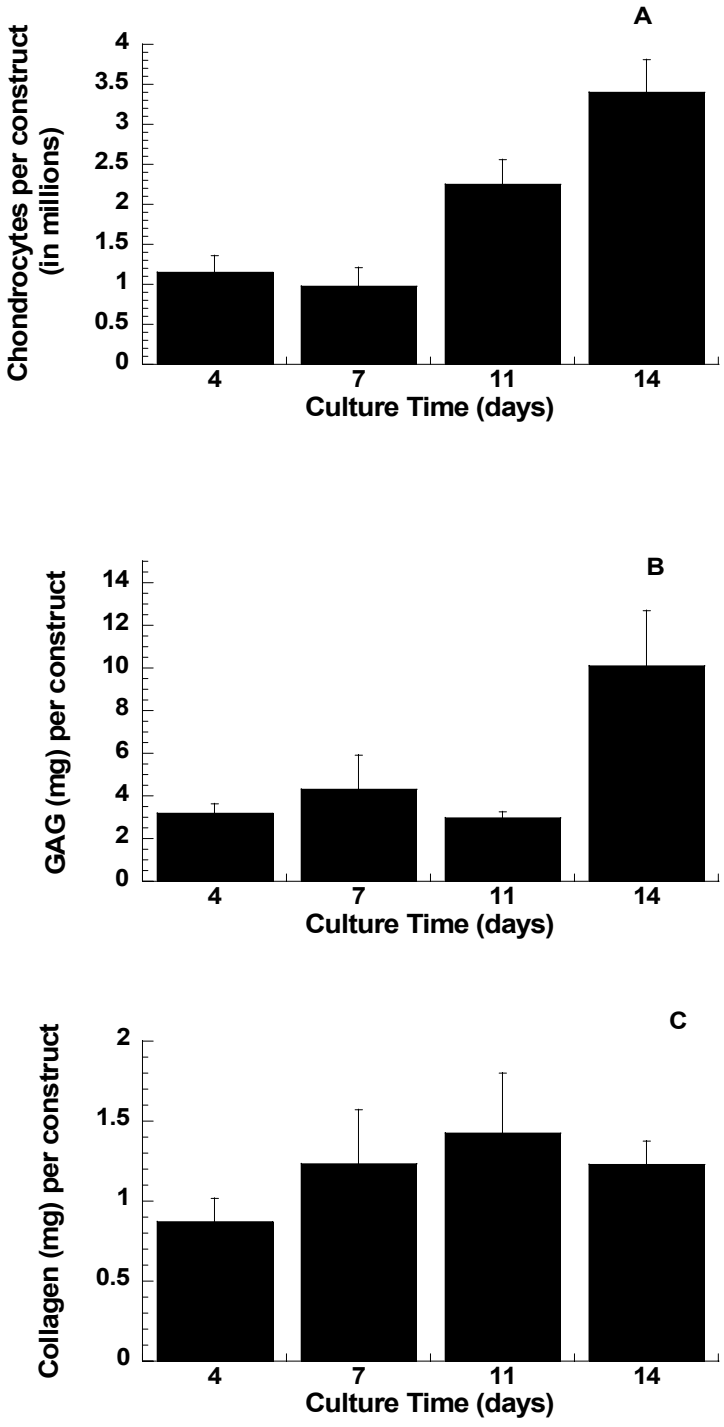


Figure 26: Short term construct development in the perfusion bioreactor: 14 day studies showing (A)construct cell number, (B)GAG per construct (mg) and (C) collagen per construct (mg). Data are mean ± SEM from N=4 experiments, n=12-14 constructs.

Long Term Construct development (35 days)

To assess the growth of cartilage constructs long term in the perfusion concentric cylinder bioreactor, one long term 35-day study was carried out. Figure 27 shows a photograph of the cartilage construct taken immediately after harvest on day 35. The construct has a glossy appearance characteristic of cartilage.

Three constructs were harvested at each time point and analyzed for bulk composition. Robust cell proliferation was observed over the 35-day period. On day 4, the constructs had 0.5 ± 0.3 million chondrocytes per construct and this increased to 7.2 ± 0.13 million chondrocytes per construct on day 20 (Figure 28A). There was a slight decrease in construct cell population between days 20 and 35. Construct collagen content was of the order of 1 mg per construct even after 35 days in culture (Figure 28C). Construct GAG content was 6.5 mg per construct on day 35 indicating that GAG production in the perfusion bioreactor was robust (Figure 28B).

Construct material properties were measured for 35-day constructs using both compression and shear testing. Construct shear modulus was 33 kPa and compression modulus was 51 kPa (Figure 29). Representative stress relaxation curve and linear regression is shown in Figure 30. The stress relaxation curve and the high coefficient of regression (R^2) confirm that within the range of strains selected, the response of the constructs obeys the Hooke's law.

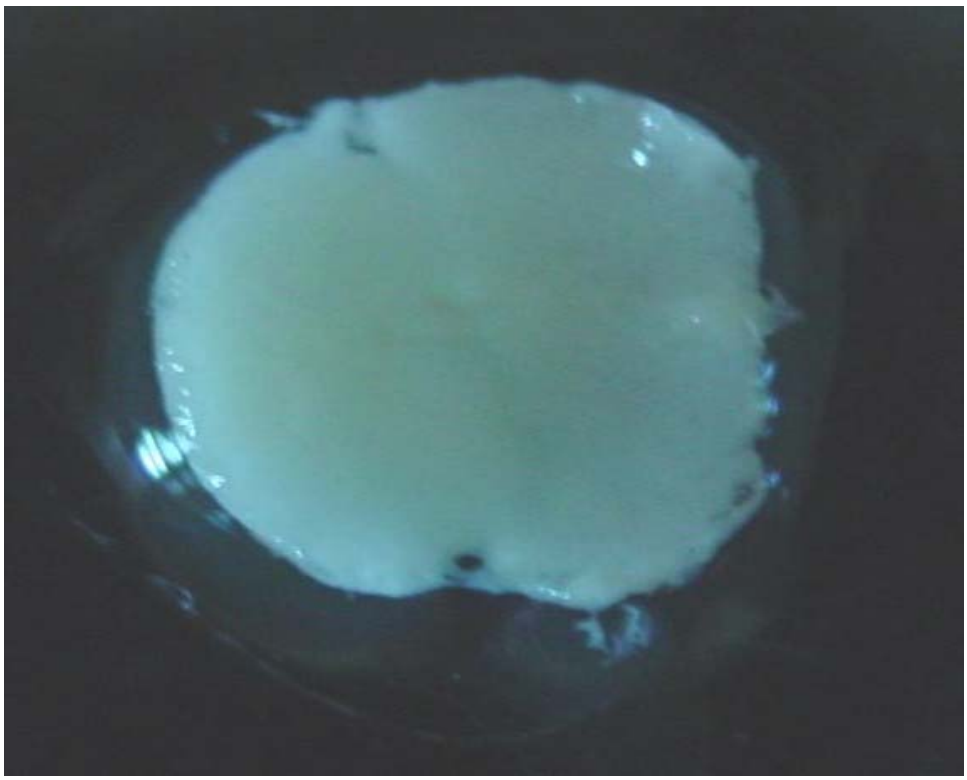


Figure 27: Photograph of tissue engineered cartilage construct harvested from the perfusion shear flow bioreactor after 35 days in culture. The appearance of the construct is glossy similar to native cartilage.

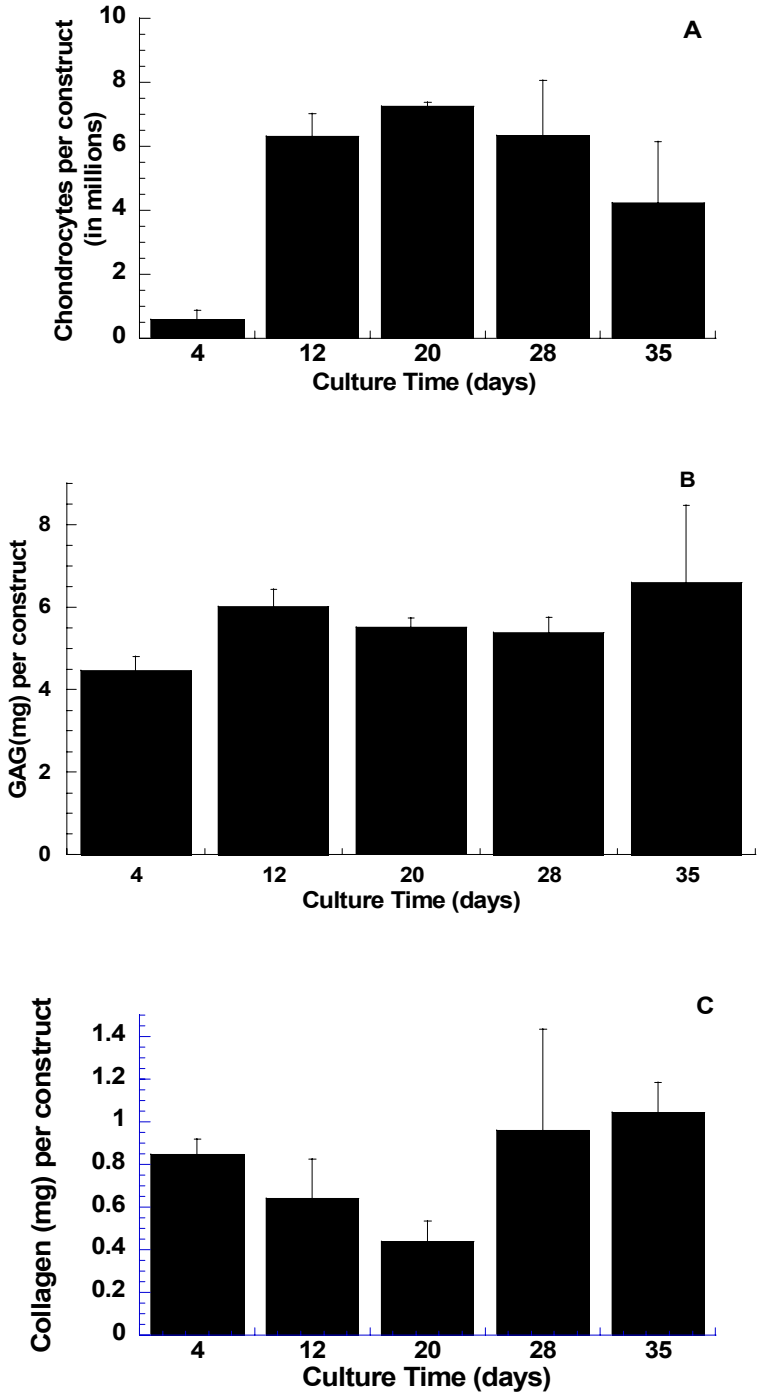


Figure 28: Long term construct development in the perfusion bioreactor. Data are from n=3 constructs from one experiment. (A) Chondrocytes per construct (in millions), (B) GAG per construct (mg), (C) Collagen per construct (mg)

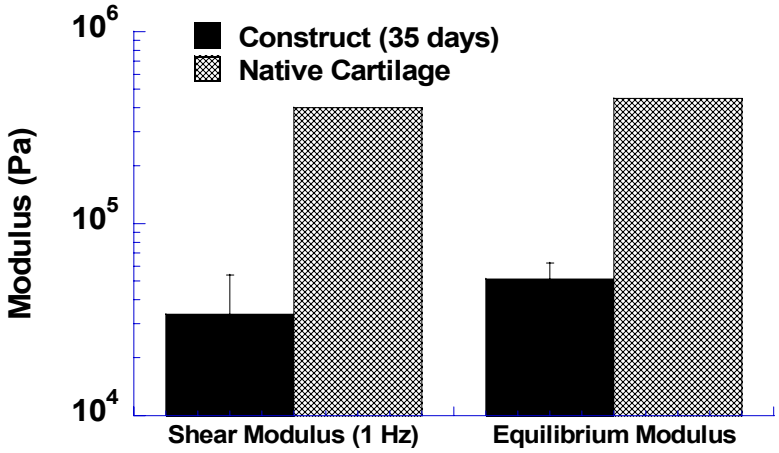


Figure 29: Material properties of the construct. Construct equilibrium modulus and shear modulus are shown after 35 day culture in the perfusion bioreactor

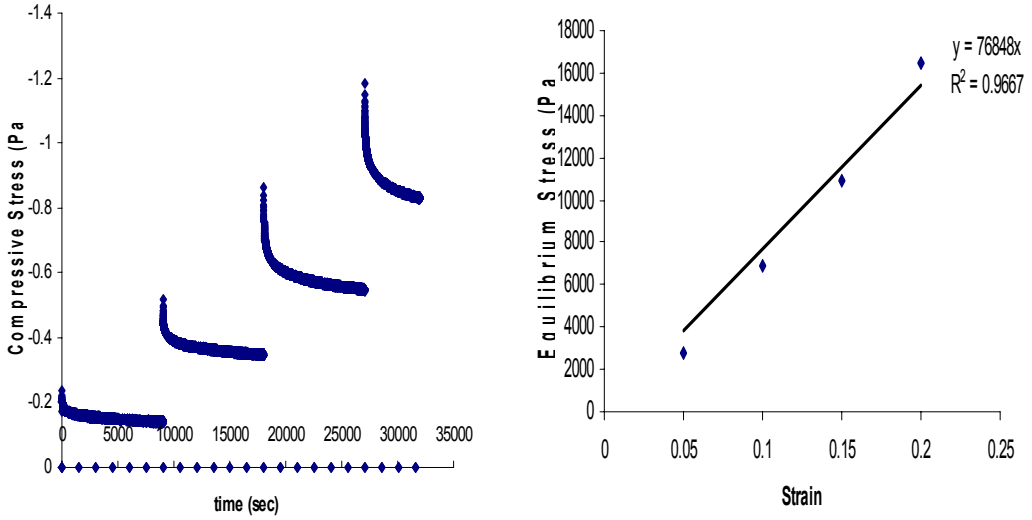


Figure 30: Representative stress relaxation curves and linear regression to obtain the equilibrium modulus of the 35 day construct.

Summary

These studies examine the effect of fluid induced shear stress (in varying hydrodynamic loading and perfusion flow) and low oxygen tension on tissue engineered cartilage construct development. As opposed to constructs cultured under varying hydrodynamic loading or low oxygen tension alone, combining both these conditions increased construct collagen content and dynamic shear modulus significantly. In order to incorporate multiple fluid flow regimes, a perfusion shear flow bioreactor has been designed and used to grow cartilage constructs. Preliminary results show that this bioreactor is capable of supporting chondrogenesis and is a potential tool for the development of functional cartilage constructs.

DISCUSSION

Joint cartilage can withstand an astonishing amount of load and physical stress per tissue volume. However, when the tissue is damaged, it exhibits limited capacity for self-repair. Adult cartilage is anisotropic both in terms of cell and matrix morphology and material properties. Formation and development of cartilage in the embryo is directed by an array of growth factors and signaling pathways in a uniquely timed sequence on specifically positioned and/or patterned mesenchymal cells. From a tissue engineering perspective, the current state-of-the-art for in vitro construct development is the culture of cells seeded on a biodegradable polymer and cultured under stimulatory conditions (presence of growth factors, compressive loading, shear stress, perfusion, hydrostatic pressure etc.). The end objective of cartilage tissue engineering is to target patients with osteoarthritis and offer a clinically relevant repair and regeneration process for already damaged joints (Caplan, 2003).

The molecular structure of cartilage is the key to material properties and behavior of tissue-engineered cartilage. This raises issues such as collagen content of the tissue and the molecular weight of proteoglycans that are laid by the chondrocytes in the tissue construct. The proteoglycans cannot function without the collagen or vice-versa. Rapid chondrogenesis can be achieved only if the native environment of adult articular cartilage is manipulated so that the deficiencies inherent to slow healing are overcome. The use of primary

chondrocytes seeded at high population has been successful in achieving rapid integration of tissue engineered grafts to native tissue (Lohmander, 2003).

In this work, we have studied using primary bovine chondrocytes and PLLA, the effects of shear stress, shear stress and oxygen tension and shear stress in the presence of perfusion in an attempt to understand the effect of culture conditions on chondrocyte extracellular matrix deposition. Specifically, the effect of hydrodynamic loading in two separate loading schedules was examined. The effect of combined low oxygen tension with hydrodynamic loading was studied to increase construct extracellular matrix properties. To incorporate multiple flow regimes, a perfusion concentric cylinder bioreactor has been designed and validated for the development of tissue engineered cartilage constructs with flow-induced alignment of cells.

Effect of low oxygen tension on construct development

Culture of tissue engineered cartilage constructs under low oxygen tension in the concentric cylinder bioreactor for 35 days showed an increase in construct collagen and GAG content, compared to normoxic cultures (Figure 10). While the collagen content was similar in either oxygen tension at day 24, there was an increase in construct collagen content at day 35. There was no change in construct cell number in either oxygen tension.

Comparing 5 and 20% oxygen tension in 22-day bioreactor cultures in the concentric cylinder bioreactor, the GAG production increased two-fold in tissue engineered cartilage constructs (Saini and Wick, 2004). There was no change in construct cell number or collagen synthesis in the low oxygen tension culture as

compared to 20% oxygen tension. In the current study, longer culture times showed an increase in construct collagen also, indicating that low oxygen tension is a favorable environment for long term culture of cartilage constructs.

These results are not completely unexpected for chondrocytes because low oxygen tension is a familiar environment for cartilage. The diffusion of oxygen is limited to <200 μm away from the blood vessel in the tissue. Cartilage is about 1-3 mm away from a nutrient artery on the growth plate end and is surrounded by synovial fluid at the articulating surface. Therefore, oxygen tension in native cartilage is low. Thus, low oxygen tension is a key player in chondrocyte metabolism in vivo. As seen in the present study, applying low oxygen tension in vitro, enhances construct extracellular matrix deposition.

These results are supported by hypoxic culture studies in explants and monolayer cell culture. Periosteal explant from rabbits were cultured at 1, 5, 15 and 90% oxygen tension to study the role of oxygen tension in articular cartilage repair in explant culture. The best results of chondrogenesis were obtained at 15% oxygen tension, with maximal cartilage and type-II collagen formation. Cartilage formation in the entire periosteum was maximal in the range of 12-15% oxygen tension (O'Driscoll et al., 1997) and not in an anoxic or hyperoxic environment. Thus, better chondrogenesis is observed in a moderately hypoxic environment.

The effect of a range of oxygen tensions, from 1-90% was studied on chondrocyte DNA and matrix synthesis in explant culture (Ysart and Mason, 1994). In cultures incubated at 24% oxygen, GAG synthesis was maintained at a

steady elevated rate throughout the seven day period of the study. Long term data was not available for this study, preventing us from drawing direct comparisons with our data.

The oxygen tension dependence of bovine articular chondrocytes was studied in alginate bead culture. This study showed that chondrocytes cultured under 10% oxygen tension had highest pellet volume when compared with 0.1, 5 and 20% oxygen tension (Grimshaw and Mason, 2000). Proteoglycan aggregation was higher at oxygen tensions between 3-9% oxygen tensions as compared to proteoglycan aggregation between 13-60% oxygen tension (Clark et al., 1991).

In our study, we picked an oxygen tension of 5% because the oxygen tension of cartilage in vivo is between 3-8 % and as mentioned before, oxygen tensions lower than 1% are not beneficial for extracellular matrix deposition. Comparing the current results with the literature, 5% oxygen tension has favorable effect of chondrogenesis and extracellular matrix deposition in vitro and we have used this value in further studies.

Effect of varying hydrodynamic loading on construct development

Fluid induced shear stress and interstitial fluid flow play an important role in eliminating mass transport gradients in tissue development in vitro. Additionally, fluid induced shear stress plays an important role in providing mechanical stimulation to chondrocytes to enhance matrix deposition in the constructs. Shear stress studies on chondrocytes have focused on maintaining the same level of shear throughout the culture period. Changing the shear stress

over the culture time as in oscillating shear stress mitigated the loss of GAG from the constructs in the concentric cylinder bioreactor (Saini and Wick, 2003). A challenge for cartilage tissue engineering is identifying and understanding the nutrient and mechanical loading conditions that optimize tissue production. Previous studies in the concentric cylinder bioreactor have shown that chondrocyte matrix deposition is a complex function of steady shear stress, with lower shear forces favoring collagen deposition whereas GAG accumulation is higher at higher shear loading (Saini and Wick, 2003). Oscillatory loading between higher and lower shear mitigates the ~60% drop in matrix GAG deposition. Based on this prior information, hydrodynamic loading schedules have been devised for varying shear stress on construct surface. By using decreasing hydrodynamic loading, it has been shown that sequentially decreasing the hydrodynamic loading during culture leads to ~40% increase on a per construct basis in matrix GAG deposition in long term culture (Figure 11B). In contrast, the construct collagen content was higher in the constructs cultured under 76 rpm by ~ 50% as compared to constructs under decreasing hydrodynamic loading. When the reverse order of shear stress was followed, as in the increasing hydrodynamic loading schedule, the GAG levels in the construct at steady rotation of 19 rpm reached similar levels as the DHL but the collagen levels were lower at all time points.

The justification for varying shear stress during culture is supported by cell signaling studies. As opposed to constant shear stress on construct, it has been shown that long-term intermittent shear deformation improves the cartilaginous

tissue formation in vitro. Shear stress at constant levels increases the expression of inflammatory cytokines such as TIMP-1, IL-6 and MMPs, suggesting that long-term exposure to shear stress at constant levels is unfavorable for cartilage development in vitro. This idea is supported by studies where application of long-term intermittent shear stress in fact promotes cartilage matrix synthesis (Waldman et al., 2003b). Direct application of low amplitude shear forces increased expression of collagen and not proteoglycans in cartilage explants. After 4 weeks of cyclic shear stress, collagen content increases by 40% and proteoglycan content increased by 35% in 3-D constructs with bovine chondrocytes. Tissues formed in the presence of shearing forces also displayed a 3-fold increase in compressive load bearing capability and 6-fold increase in stiffness.

Thus, varying the hydrodynamic loading or outer cup rotation in the concentric cylinder bioreactor was used to incorporate the above ideas of intermittent biophysical stimulation. The decreasing hydrodynamic loading regimen was chosen based on the GAG accumulation kinetics presented in (Saini and Wick, 2003). We expected to see an increase in construct GAG and collagen contents in the decreasing hydrodynamic loading regimen as opposed to the steady shear of 76 rpm. However, the GAG increase was only about 40% on a per construct basis and the decreasing loading did not increase construct collagen content. This could be possibly be due to a complex response of the chondrocytes to the biophysical stimuli. In contrast to the decreasing hydrodynamic loading, the increasing hydrodynamic loading did not show an

increase in GAG when compared to the steady control (19 rpm). These results seem to suggest that the chosen stimuli and loading regimens may perhaps not be the most effective ones for increasing construct extracellular matrix production. In addition, the response of the chondrocytes to the stimuli may be complicated, making it hard to discern differences at a macroscopic level.

In monolayer bovine chondrocyte culture, fluid induced shear stress stimulated GAG synthesis by 2-fold and increased the length of the newly synthesized chains. Dynamic shear stress on cartilage explants increases protein synthesis and proteoglycan significantly above the levels of statically cultured explants (Jin et al., 2001). Shear stress at levels of 1.52 dyn/cm^2 increased GAG production by 2-fold in constructs with bovine chondrocytes after 5 days of culture. Nitric oxide release increased with the duration and magnitude of applied shear stress (Smith et al., 1995). Shear stress also increased the expression of interleukin-6 and tissue inhibitor of metalloproteinase-1 (TIMP-1). A shear stress of 16.4 dyn/cm^2 applied on human osteoarthritic chondrocytes cultured in monolayer increased NO release up to 3.5-fold over 24 hours as compared to static control. Shear induced NO directly influenced regulation and coordination of the biomechanical responses of chondrocytes by altering matrix mRNA signal levels. Inhibiting NO production significantly altered and reversed the inhibitory effects of shear stress on osteoarthritic chondrocytes (Lee et al., 2002). These results suggest that shear stress regulation of NO plays an important role in the response of chondrocytes to shear stress.

While the elucidation of specific signaling pathways was not the objective of this work, it should be noted that numerous signal transduction pathways are affected by shear stress. Signaling mechanisms for detecting change in shear stress or loading possibly include stretch activated ion channels and integrin-cytoskeleton machinery which can trigger kinase cascades leading to changes in protein expression and transcriptional regulation. Mechanical stresses may also affect chondrocyte synthesis of extracellular matrix by changing the structure of organelles such as endoplasmic reticulum and the Golgi apparatus (Grodzinsky et al., 2000). Chondrocytes respond favorably to dynamic mechanical stimulation by up-regulating proteoglycan synthesis. Long term intermittent shear application and biomechanical conditioning on statically cultured constructs showed increased proteoglycan deposition (Waldman et al., 2003b; Waldman et al., 2003a). One possible explanation for the response of chondrocytes to varying shear stress is that high initial stimulation increases matrix molecule biosynthesis. This may also explain the differences in the IHL and DHL schedules.

There was no observed increase in the collagen content of the constructs cultured in the decreasing or increasing shear stress environment over the culture period. The constructs cultured in 76 rpm environment indicated an increase in construct collagen content after 35 days (Figure 11C). Thus, the response of chondrocytes to a varying shear stress regimen is complex and multiple biochemical pathways may be affected by a single shear stress loading regimen.

Two different hydrodynamic loading regimens were studied in this work. However, it is possible that intermittent application of high shear stress as opposed to continuous application (akin to intermittent dynamic stimulation) may hold the key to enhancing matrix deposition in the constructs.

Effect of low oxygen tension and varying shear stress

The bioreactor conditions outlined in the previous studies along with published results for the concentric cylinder bioreactor show that extracellular matrix deposition is a complex function of hydrodynamic loading (Saini and Wick, 2003) and oxygen tension (Saini and Wick, 2004). While these conditions stimulate GAG deposition, no significant changes in construct collagen content are observed. The current study demonstrates that culture under hypoxia with decreased hydrodynamic loading dramatically increases collagen deposition and improves material properties of tissue engineered cartilage constructs in bioreactor culture.

After 35 days in bioreactor culture, construct collagen content in the hypoxic environment with decreased hydrodynamic loading is 3-fold higher than the construct collagen content in the normoxic environment (Figure 17C). Type II collagen is higher in 5% oxygen tension (Figure 18). These results are in agreement with previous studies which show that, under hypoxic culture conditions, chondrocyte collagen II mRNA levels (Grimshaw and Mason, 2001), collagen II mRNA transcription (Murphy and Sambanis, 2001b; Maroudas, 1980), and collagen II protein secretion (Hansen et al., 2001; Domm et al., 2002) increase with a decrease in oxygen tension in monolayer or aggregate cultures in

alginate beads. Thus, combining decreasing hydrodynamic loading with low oxygen tension increases construct extracellular matrix deposition than either condition alone.

Construct glycosaminoglycan content does not vary significantly between 12 and 35 days in both hypoxic and normoxic bioreactor culture conditions, although at the end of culture, constructs cultured under decreased hydrodynamic loading in 5% oxygen tension have 40% higher GAG content compared to constructs cultured under decreased hydrodynamic loading in 20% oxygen tension. These data suggest that the effect of low oxygen tension on matrix GAG synthesis is most prominent early in bioreactor culture. The increase in GAG accumulation in constructs cultured in 5% oxygen tension may also result from better organization and incorporation of the small molecular weight proteoglycans in the extracellular matrix under hypoxic conditions. This observation is consistent with previous data suggesting that low oxygen tension environment increases the aggregation of newly synthesized proteoglycans (Clark et al., 1991). The increase in GAG production early in bioreactor culture combined with possible aggregation of proteoglycans suggest that hypoxic environment can reduce bioprocessing time for cartilage tissue engineering in vitro.

It is important to recognize here that decreasing hydrodynamic loading and low oxygen tension by themselves did not exhibit a large increase in construct collagen content, and showed only small increases in construct GAG content. However, the combination of these stimuli seems to be synergistic and

leads to a large increase in construct collagen deposition and increases construct GAG deposition even early on in culture. This effect may be attributed to multiple signaling pathways. Some of the possibilities are presented here as speculation alone. Ascorbic acid stimulates collagen production and the low oxygen tension environment may retain/promote the activity of ascorbic acid and reduce the activity of matrix metalloproteinases. This action would allow for increased deposition of construct collagen content. Matrix metalloproteinases are known to trigger degradation of cartilage matrix. In addition, they are also known to induce aggrecanase activity. Therefore, the low oxygen tension environment may be inducing an environment that allows the hydrodynamic environment to enhance matrix deposition, thereby leading to synergistic effects.

Constructs cultured in hypoxia with decreased hydrodynamic loading have a higher dynamic shear modulus as compared to constructs cultured in normoxia with decreased hydrodynamic loading. After 35 days of bioreactor culture, the shear modulus of constructs in hypoxia with decreased hydrodynamic loading culture is between 247 and 345 kPa (Figure 19) and within the range of 200-400 kPa reported for native cartilage (Ratcliffe and Mow, 1996). This increase in shear modulus in constructs containing more collagen suggests that bioreactor conditions that increase construct extracellular matrix content and organization lead to increases in viscoelastic properties of the construct. This is consistent with previous reports that an increase in construct collagen content of 40% increases the compressive load bearing capacity 6-fold and stiffness 3-fold of engineered tissue formed in vitro (Waldman et al., 2003b). These studies

indicate that the construct material properties depend on the construct matrix content.

One of the physiologic functions of articular cartilage is to serve as a reservoir of elastic energy during locomotion, which saves muscular work and protects cartilage from fibrillation and fissure formation (Silver et al., 2002; Silver and Bradica, 2002). The energy storage capability is decreased or impaired in fibrillated or fissured tissue as is the case in osteoarthritis. In arthritic joints because of the friction due to rough articulation and abraded joints, there is high energy loss due to joint motion. This increases the strain on the muscle during motion and causes pain. Identifying the mechanical function of the collagen network in native cartilage allows us to define a 'functional' goal for tissue engineering. While measuring construct biochemical composition and material properties are established procedures of evaluating construct quality, the energy functions of the construct allow us to compare the construct directly to native cartilage in terms of functionality and performance.

Based on the shear modulus data, the elastic energy stored per unit volume and the viscous energy dissipated per unit volume of the scaffold is shown in Figure 31. The detailed derivations are shown in Appendix (1). The constructs with higher collagen content (5% oxygen tension) have higher elastic energy storage than the constructs with lower collagen content (20% oxygen tension). The viscous energy loss is higher for constructs grown under 20% oxygen tension at lower frequency and drops with frequency. The viscous energy loss is lower for constructs cultured under 5% oxygen tension and is

essentially frequency independent. The high elastic modulus of articular cartilage suggests that one of the molecular functions of collagen in cartilage is to store elastic energy during locomotion. In this case, it is easy to see that constructs with more ECM have higher energy performance.

Correlations between construct shear modulus and construct collagen content are important to understand the structure-function relationship between construct biochemical composition and material properties. Extensive information is available on the material properties of native tissue and different groups have proposed models to explain the viscoelastic behavior of cartilage (Mow et al., 1984; Mow and Wang, 1999; Zhu et al., 1993). Shear modulus depends strongly on the tissue collagen content in ovine (Appleyard et al., 2003) and bovine cartilage (Zhu et al., 1993). For cell-polymer cartilage constructs (bovine chondrocytes in poly glycolic acid scaffolds) the complex shear modulus depends approximately quadratically on the construct collagen content (Stading and Langer, 1999).

In order to determine the shear modulus of polymer constructs alone, unseeded PLLA constructs were soaked in culture medium for 35 days and their viscoelastic properties measured. Unseeded PLLA scaffold had viscoelastic properties in the same range as that of the construct (Figure 20). PLLA is a highly crystalline polymer and degrades via random, bulk hydrolysis of the ester bonds. The oligomers closer to the surface degrade faster than the oligomers inside the scaffold. This leads to a delay in the decrease of the mechanical properties of the polymer. PLLA remains stable for over a year in the body.

PLLA takes from 9-15 months to register loss in molecular weight and mechanical properties (Hutmacher, 2001; Hutmacher, 2000) and is used extensively in orthopedic, oral and maxillofacial surgery. Because of its slow degradation time in the body, PLLA is an extensively used biodegradable polymer.

Although unseeded PLLA scaffold had material properties in the same range as the constructs, in response to the testing procedure, the scaffold disintegrated. The disintegration of the scaffold at the end of the testing and decrease in shear modulus with the frequency indicated possible damage to the microstructure of the scaffold. This is supported by results from other studies where after 6 weeks of being soaked in phosphate buffered saline under static conditions, PLLA scaffolds with different porosities were too fragile for mechanical property testing (Agrawal et al., 2000). Digestion of the cartilage construct in papain at the different times (days 12, 24 and 35) revealed very little to no polymer residues in the tissue construct in bioreactor culture. This suggests that the dynamic shear modulus G* of the construct was from the presence of ECM in the constructs and not polymer alone.

To investigate the nature of the relationship between collagen content and the dynamic shear modulus for constructs cultured in this study, regression analysis was performed between the two quantities (Figure 32). The analysis was performed separately for 5% oxygen tension with decreased hydrodynamic loading and for 20% oxygen tension with decreased hydrodynamic loading. Different models including linear, second order polynomial, and exponential were

used to correlate the construct collagen content with the shear modulus. Constructs cultured in 5% oxygen tension with decreased hydrodynamic loading showed better fits for the tested models compared to the constructs from 20% oxygen tension with decreased hydrodynamic loading environment. No correlations were found for constructs cultured in normoxic environment. The R^2 values were 0.33 and 0.32 for the exponential and quadratic models respectively. In the small range of collagen content produced in tissue engineered cartilage, the behavior of exponential and quadratic models is mathematically similar. Unlike collagen content, construct GAG content did not exhibit a significant correlation with the shear moduli of constructs. Similarly, construct shear modulus did not correlate with total construct matrix.

About 60-80% of the extracellular matrix in native cartilage is collagen II, with the balance of the matrix being GAG. Collagen is primarily responsible for the mechanical integrity of cartilage (Riesle et al., 1998), so it is not surprising that an increase in collagen content leads to an increase in G*. The swollen state of the collagen network due to the proteoglycan fixed charge density not only provides a pre-stressed state for tissue equilibrium, it also allows for load bearing functions.

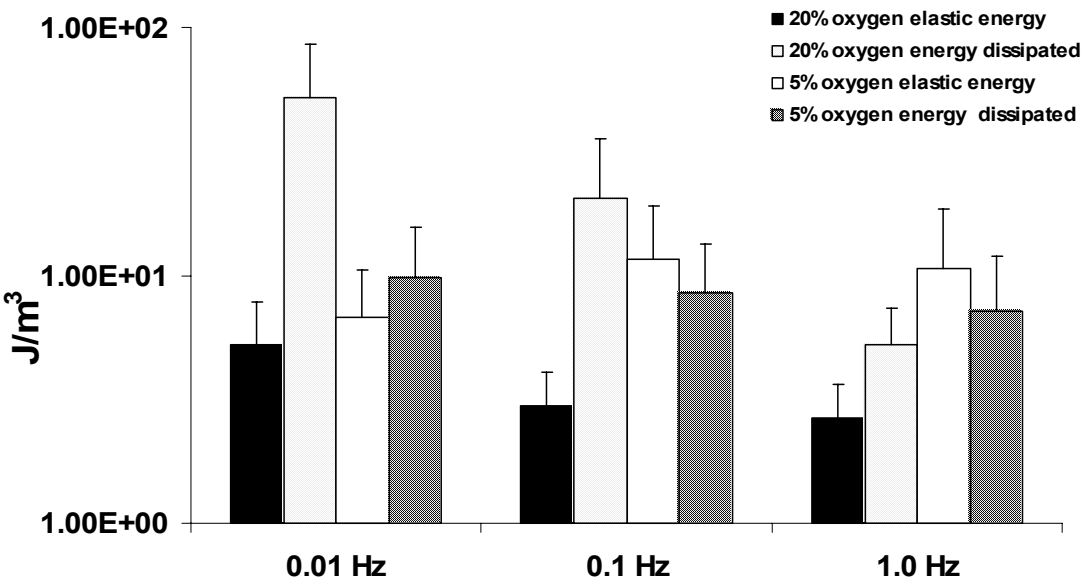


Figure 32: Elastic energy storage and viscous energy dissipation within the constructs.

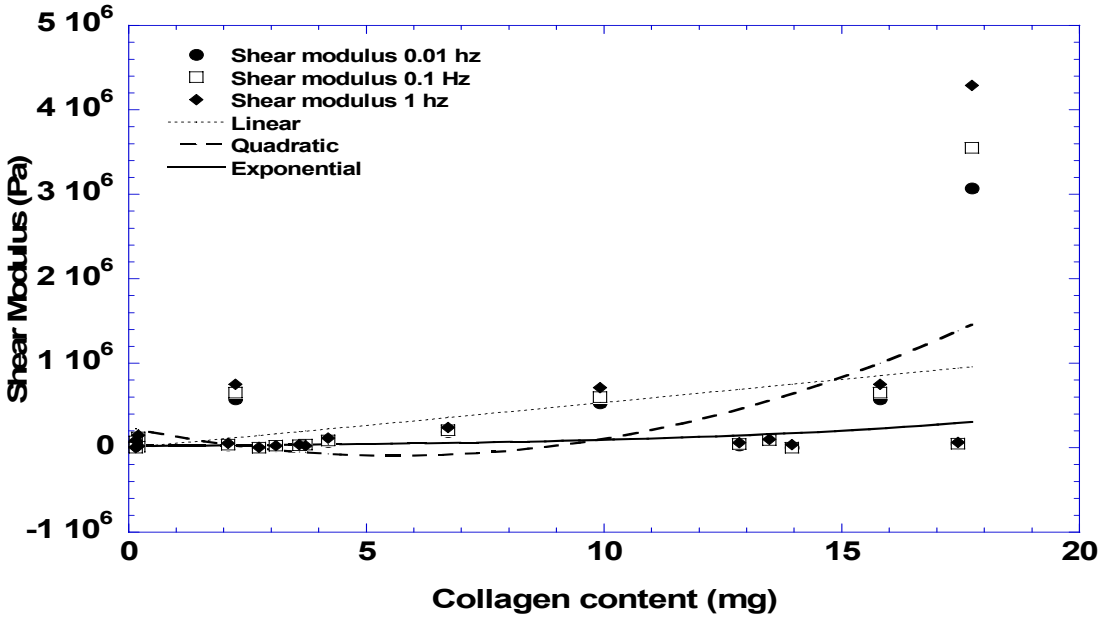


Figure 33: Regression analysis for constructs cultured under 5% oxygen tension with DHL.

Thus, the interaction between collagen fibers and GAG molecules is multipart and likely contributes to the overall mechanical behavior of constructs. This is also supported by observation of an inverse correlation between proteoglycan content and dynamic shear modulus in the ovine articular cartilage model (Appleyard et al., 2003) and by data demonstrating that the dynamic shear modulus of cartilage decreases considerably when the proteoglycans are degraded (Zhu et al., 1993).

Controlled application of mechanical loading and oxygen transport on chondrocytes in bioreactor culture increases matrix deposition in cartilage tissue. This indicates that the most favorable bioprocessing conditions may be a combination of previously known bioreactor conditions.

Effect of TGF-β1

Members of the transforming growth factor – β (TGF-β) superfamily are thought to play a key role in chondrocyte growth and differentiation. In one bioreactor experiment, addition of 0.2 ng/ml of TGF-β1 to complete DMEM increased construct GAG content and cell proliferation two fold in 24 days as compared to cultured without DMEM (Figure 22).

The use of TGF-β1 in culture has diverse effects based on the culture environment. TGF-β has a stimulatory effect on cartilage explants on proteoglycan production and cell proliferation. TGF-β also plays an important role in preventing cartilage matrix degradation in the presence of inflammatory cytokines such as IL-1 (Grimaud et al., 2002).

Our observation that TGF- β1 increases chondrocyte proliferation early in culture (Figure 22A, Figure 23A) is in agreement with proliferation studies of chondrocytes in the presence of TGF-β1 early (5 days) in culture (Kim et al., 2003). However, not much is known about the action of TGF-β1 in long term 3D chondrocyte culture. It has been shown that collagen accumulation can down-regulate the activity of TGF-β1 (van der Kraan et al., 2002) in chondrocyte gel cultures. Similarly, in alginate encapsulated chondrocyte cultures, 0.1 ng/ml TGF-β1 initially increases some proteoglycans like keratan sulfate but as collagen production increases, the sensitivity of the cells to TGF-β1 decreases (Qi and Scully, 2003). The proteoglycans decorin, biglycan, and fibromodulin regulate the activity of TGF-β proteins by sequestering TGF-β within the extra cellular matrix; suggesting inhibition of TGF-β activity after binding to small proteoglycans (21). The high initial cell proliferation in bioreactor culture (Figure 24A) may be explained by the active TGF-β1 in the media promoting chondrocytes to divide rapidly and deposit extracellular matrix early in culture. However, it is possible that as the quantity of extracellular matrix increases, the activity of TGF-β decreases later in culture.

Construct GAG deposition (Figure 23B) decreased over time in the presence of TGF-β1. This suggests that long term exposure of chondrocytes to TGF- β1 in bioreactors is detrimental to construct development. This is in agreement with observations that short term treatments with intra-articular injections of TGF-β for patients with osteoarthritis showed favorable results. However, long term intra-articular injections disrupts cartilage homeostasis

(Grimaud et al., 2002). Thus, the beneficial effect of TGF-β on tissue growth and repair may be counteracted by tissue degradation seen with longer exposure to TGF- β1 in bioreactors and in vivo (8).

Nitric oxide is believed to be a marker for potential osteoarthritis (Studer et al., 1999b). Increased levels of nitrate/nitrite accumulation in the conditioned media from the bioreactor are indicative of NO production by the chondrocytes. Increased nitric oxide production by chondrocytes has been observed in the presence of TGF-β1 (Vodovotz, 1997). Nitric oxide and transforming growth factor proteins have a complex mechanism of action on chondrocyte metabolism (Lane et al., 2000; Vodovotz, 1997). It has also been shown that nitric oxide can modulate proteoglycan synthesis indirectly by decreasing the production of TGF-β1. Nitric oxide appears to interfere with the autocrine mechanisms of TGF-β1 and may potentially diminish the anabolic effects of the growth factor on chondrocyte metabolism (Studer et al., 1999a). Although the mechanism of nitric oxide and TGF-β1 is not completely understood, it is possible that these interactions play a major role in diminishing matrix GAG deposition in the bioreactor. The decrease in chondrocyte proliferation in the presence of TGF-β1 over the culture period coupled with increased nitrate/nitrite accumulation suggests a specific cell behavior, which indicates that the exposure of chondrocytes to TGF-β1 needs to be regulated over the culture period.

The effect of mechanical and biochemical conditioning is not necessarily additive in bioreactor culture as multiple stimuli and pathways can affect chondrocyte response. In this study we found that sequential decrease in

hydrodynamic loading increases matrix GAG deposition but in the presence of sequential decrease in hydrodynamic loading and TGF-β1, the matrix GAG produced after 35 days in culture was lower than that found in sequential decrease in hydrodynamic loading alone. The differential response of chondrocytes to shear stress and growth factor can be exploited in a bioreactor to modulate chondrocyte proliferation and matrix deposition. Exposure to TGF-β1 early in culture to promote chondrocyte proliferation, followed by removal of TGF-β1 from culture media and sequential reduction in hydrodynamic loading to stimulate matrix deposition appears to be favorable for cartilage construct development in bioreactors. The concentric cylinder bioreactor provides a dynamic environment regulating biochemical and mechanical stimuli to enhance cartilage construct development.

In summary, combining decreased hydrodynamic loading with low oxygen tension culture increases construct extracellular matrix deposition and enhances material properties in tissue engineered cartilage constructs in the concentric cylinder bioreactor. One goal of fundamental tissue engineering research is to develop predictive models of tissue growth for different culture environments to identify bioreactor conditions that enhance tissue development. The present studies and analyses demonstrate that the concentric cylinder bioreactor is an effective tool for cartilage tissue engineering to understand the development of cartilage constructs with enhanced material properties for potential in vivo application.

Perfusion concentric cylinder bioreactor

The primary design objective of the perfusion concentric cylinder bioreactor was incorporation of axial and radial shear stress in the constructs to provide – (a) mechanical stimulation to cells inside the construct, (b) minimal nutrient gradient through the construct thickness, (c) cell alignment in the construct – parallel along the outer surface, perpendicular inside the construct. Additionally, the bioreactor was designed in such that way that scale-up and preservation of the constructs for downstream processing would be possible. These ideas are based on the need to produce cartilage constructs that are functionally relevant for in vivo performance. Existing bioreactor designs such as the spinner flask, rotating wall vessel bioreactor, and perfusion bioreactors have been described in detail in the background. In our lab, Dr. Saini had designed the concentric cylinder bioreactor to address the requirements of tissue engineering such as (a) uniform hydrodynamic loading, (b) large growth area to accommodate multiple constructs, (c) simple and scalable geometry. All of these bioreactors have demonstrated that cell-seeded polymers form cartilaginous tissue and tissue morphology and extracellular matrix content is dependent on the mixing and flow conditions in the bioreactor.

Perfusion bioreactors specifically have been designed and utilized successfully for cartilage growth by perfusing culture medium through the inner porous structure of the scaffolds thereby providing enhanced nutrient transport and mechanical stimulation to the cells seeded inside the construct (Davisson et al., 2002b; Ratcliffe and Niklason, 2002). These bioreactors offer a specific

advantage of housing each construct in its separate casing and thereby making it easy to package and preserve after the culture period. A major concern cited for this bioreactor is that as the tissue develops and more extracellular matrix is laid, there will be more resistance to fluid flow through the construct, leading to increased pressure drop across the tissue. This could lead to the formation of a necrotic core in the middle of the construct.

The current bioreactor design alleviates this by having low shear stress environment in the construct and having both perfusion and surface shear to mitigate nutrient deficiencies. As extracellular matrix is deposited and the porosity of the construct decreases, the perfusion flow rate can be decreased or even completely stopped to prevent pressure build-up. Because perfusion is not the only source of fluid flow, the inner part of the construct will continue to receive nutrients because of the presence of surface shear stress. This concept can be exploited to provide different nutrients or growth factor cocktails on the two different sides of the construct as the tissue matures. One obvious example would be to provide factors that provide osseous factors on the perfusion end of the construct and cartilage promoting factors at the outer cup end of the construct.

The complex ultrastructure of native articular cartilage has been described previously. The zonal variation of cellular alignment and fibril alignment provide cartilage with its unique load bearing capabilities and material properties. For tissue engineered cartilage in bioreactors, it takes up to 7 months in a spinner flask to attain compressive load bearing capabilities similar to that of native tissue

(Vunjak-Novakovic et al., 1999; Vunjak-Novakovic et al., 2002). By incorporating fluid flow regimes in the perfusion concentric cylinder bioreactor that allow for cellular and fibril alignment, we envision to reduce this culture time and also tissue engineer constructs that have a spatio-temporal variation within the constructs.

The perfusion concentric bioreactor allows for dynamic seeding of constructs. The purpose of dynamic seeding is to establish a system where multiple constructs can be seeded almost identically under flow conditions. Dynamic seeding allows for minimal handling of the tissue constructs and therefore contamination. The seeding phase was for four days without perfusion flow in the seeding experiments. This was because seeding using perfusion flow would involve flow of the cells through the tubing and the roller pump and this could potentially kill the cells. On day 4, about 10-15% of cells added to the bioreactor outer cup were attached to the scaffolds (Figure 22). This could be because of two reasons – (a) the large volume of media in the outer cup, (b) the vertical direction of orientation of the bioreactor. In further modifications of the bioreactor design, seeding could be done through the inner perfusion tube rather than through the cup to promote cell attachment.

Monitoring the cell content in the constructs during the seeding phase shows that the number of cells attached on days 2 and 3 are similar and there is a slight decrease in cell number on day 4 (Figure 22). Live/dead analysis of the constructs after 4 days showed a uniform distribution of cells through the

constructs (Figure 23). These data indicate that even though the seeding efficiency was low, the cells were attached to the constructs and were viable.

Short term construct development

Construct cell number increased over time in the perfusion bioreactor in the short term culture development studies. The chondrocyte number showed a 3 fold increase between days 4 and 14. Perfusion flow at 10 ml/min through the constructs was begun at day 4. The robust increase in cell number even after perfusion was begun suggested that perfusion was not causing cell detachment from the constructs after day 4.

An important step in engineering cartilage is producing constructs with appropriate chondrocyte density (Freed et al., 1999; Vunjak-Novakovic et al., 1998; Saini and Wick, 2003). In cartilage, there is a relatively low cell density (10,000 cells/cm^3) and an abundance of extracellular matrix. In order to obtain high ECM production in the tissue engineered constructs, high seeding densities have been used (Saini and Wick, 2003). In the perfusion bioreactor, the cell density after 14 days is approximately about 3.3 X 10^6 cells per construct (Figure 26A). This is less than the cell density achieved in the concentric cylinder bioreactor seeded with 100X10^6 cells per 16 constructs after 28 days, but the fold increase in cell proliferation is higher in the perfusion bioreactor (Saini, 2002; Saini and Wick, 2003).

Based on the cell proliferation data, it seems like a critical cell density would be necessary for the chondrocytes to maintain cell-cell contact and produce extracellular matrix. The environment of the perfusion bioreactor seems

to be favorable for chondrogenesis even at low cell densities. Chondrocytes seeded on PGA scaffolds showed dependence on chondrocyte initial seeding density – 5 X 10^6 cells/cm^3 did not initiate chondrogenesis whereas 10 X 10^6 cells/cm^3 allowed for chondrogenesis and extracellular matrix deposition (Vunjak-Novakovic et al., 1998).

Robust GAG deposition was observed over the short term culture period in the constructs (Figure 26B). The construct GAG content increased about 5 fold in the span of 10 days in the presence of perfusion. This supports our cell proliferation data that perfusion and surface shear may be a favorable condition for extracellular matrix deposition. The GAG deposition increased between days 11 and 14 in culture and was highest on day 14. This is in accordance with previous studies were GAG production was highest within 14 days of culture (Freed et al., 1998; Vunjak-Novakovic et al., 1998).

The construct collagen content did not show any trend over the two week culture period but seemed to remain at the same level between days 7 and 14 (Figure 27C). Multiple stimuli may be necessary to increase collagen deposition in the construct (low oxygen + DHL for example). Previous studies have shown that even in longer culture periods, collagen production remains sub-optimal. After 4-6 weeks in culture in a laminar flow bioreactor, PGA scaffolds seeded with chondrocytes had only 39% of the levels of collagen measured in native tissue (Martin et al., 2000). Therefore, it is not surprising that collagen levels were not high in the short term culture period in the perfusion bioreactor.

Kinetics of chondrocyte growth in the perfusion bioreactor

The values of the construct cell number from the seeding studies and the short term construct development studies were used to model the kinetics of cell growth in the perfusion bioreactor. Performing regression analysis on the cell number with time as the major variable shows that the cell number follows a quadratic model, which accounts for the initial cell number, initial cell growth rate and the rate of change of cell growth. The exponential model was not used in this modeling effort because (a) chondrocytes exhibit a slow growth rate, (b) the model has to account for the initial cell number in the constructs, and (c) the rate of cell growth changes with time in bioreactor culture as shown by the experimental data.

For all experiment groups, the cell growth profiles could be represented by the equation

$$Y(t) = \alpha - \beta t + \gamma t^2$$

Where Y(t) is the cell number per construct in millions at time t (in days), α, β and γ are determined from non-linear regression analysis of the data from individual experiments. α represents the initial cell number, β represents the initial cell growth rate and γ represents the rate at which cell growth rate changes. The values of the coefficients and R^2 are given in Table 10.

Table 10: Values of coefficients of kinetic models for cell growth

	Alpha	Beta	Gamma	R^2
Expt1	1.29E+06	-1.16E+05	1.80E+04	0.95555
Expt2	3.15E+06	-9.61E+05	7.69E+04	0.997
Expt3	1.76E+06	-2.47E+05	1.82E+04	0.7635
Expt4	6.25E+05	1.82E+05	4.10E+03	0.9527

The model obtained in our work is similar to models published previously (Freed et al., 1994c). These kinetic models attempt to account not only for cell proliferation but also parallel ongoing processes like ECM deposition and polymer degradation (Freed et al., 1994c). As new bioreactor design requirements are recognized and systems designed, modeling of the tissue development data allows us to minimize the number of validation studies required before the critical values such as cell density can be obtained.

Regression analysis is futile in tissue engineering for tissue growth processes if the coefficients do not have a physiological meaning. The values from the model show that the initial cell seeding density which is a critical factor for achieving the cell density in the final tissue engineered construct can be calculated based on the growth rate of the chondrocytes. Based on the experimentally observed seeding efficiency of the bioreactor system, the value of the initial number of cells to be added to the bioreactor can then be determined. The initial cell growth rate, which represents the growth rate of the isolated chondrocytes at zero time, depends on the “health” of the chondrocytes. In our experiments, we isolate cartilage from bovine knee joints and isolate the cells using enzymatic digestion. Handling and storage techniques then become critical components of cell sourcing for obtaining a cell suspension with a high initial growth rate. Chondrocytes do not proliferate endlessly, but exhibit diminished multiplication with time. The value of gamma in the regression addresses this change in chondrocyte proliferation.

One long term construct growth experiment has been carried out in the perfusion bioreactor. Robust cell proliferation and GAG deposition were observed in the bioreactor, the collagen values still remained sub-optimal. The shear modulus and the compressive modulus of the constructs were only 10% of that of native cartilage indicating that the constructs cultured in the perfusion bioreactor for 35 days were not mechanically strong. While it is hard to draw strong conclusions from one experiment, the data suggest that GAG production and deposition is upregulated by the combination of perfusion and shear flow and additional conditions such as low oxygen tension may be required to increase construct collagen production.

Experimental variability

Large variances were observed from experiment to experiment in the bioreactor studies. This made it harder to elucidate statistically significant differences and draw strong conclusions from the data sets. Some of the factors that may have affected the experiments are listed below.

Each experiment within a study was carried out using cells obtained from a different animal. The bovine knee joints were obtained from a tissue processing facility. The tissue processing facility obtained the knee joints from the slaughter house immediately after slaughter. The shipping and handling processes of the knee joints were beyond the control of the lab, until they were delivered to the facility. The standard order placed for knees was male bovine knee joints, 2-3 weeks old. However, these details were hard to verify once the knee joint was delivered to the lab. The tissue was harvested and processed

immediately and bioreactor experiments were started within 24 hours of tissue isolation. However, there could be a potential factor that introduced the variability in the experiments that may have crept in via the cell source.

Another major factor that could influence the results of the experiments is the lot of fetal bovine serum used in the culture medium. It was difficult to order the same lot of FBS over the course of all the experiments done, primarily because of availability. This may be have been reverted by ordering large quantities of FBS at the start of each study.

Differences were also observed under conditions where the results should have been similar ideally. For example, in the decreasing hydrodynamic loading studies, for the first twelve days, both bioreactors are operated under similar conditions. However, differences are seen even on day 12. While it is true that these differences were not statistically significant, it does bring up the issue of intra-experimental variability. Some sources of this could be because of bioreactor equipment, assays performed and/or because of human error in feeding and handling. Similar differences are seen on day 35, where the collagen content is higher for 76 rpm but the differences do not reach statistical significance. The large error bars prevent us from drawing definitive conclusions regarding the collagen content in the constructs. In addition, in the low oxygen tension with decreasing hydrodynamic loading has 3 experiments for the 20% + DHL condition and 4 experiments for the 5% + DHL condition. This is because in one of the experiments, there was a mechanical failure of the bioreactor being operated under 20% + DHL on day 6.

It is important to recognize that the assays used to measure the biochemical content and cell numbers are not necessarily error proof. Papain was used to digest the constructs and release the extracellular matrix components into the digestate. Papain is a very strong enzyme that could potentially damage the ECM components, preventing us from obtaining an accurate measure of construct biochemical content. Construct cell number was measured using a DNA assay was performed using a fluorometric technique where there is a large possibility for background noise, rendering the signal-to-noise ratio less than accurate. Samples had to be diluted to minimize this noise. But this leads to magnification of error because of introduction of multiplication factors to obtain the final cell numbers. Similarly, construct collagen was measured by first measuring the hydroxyproline content and then calculating the collagen content using a multiplication factor of nine. This could also lead to magnification of error sources.

It is important to note here that although there are various sources that contribute to the variability, some of the results observed in the experiments constituting the present research are more toward the exception than the rule. In many published works, researchers have shown a consistency of trends across experiments in the same study (Freed et al., 1998; Wu et al., 1999; Dunkelman et al., 1995; Carver and Heath, 1999a). In published works citing the concentric cylinder bioreactor, the authors present information on experiment to experiment variability across experiments (Saini and Wick, 2003) and it is clear that the bioreactor system has been developed and validated to produce same results

experiment after experiment. Based on the work done by the tissue engineering community, it is only appropriate to acknowledge the human error that could have potentially played a role in the experiments and analyses that constitute the current work.

CONCLUSIONS AND RECOMMENDATIONS

The current work focuses on bioreactor bioprocessing conditions to enhance the material properties and ultrastructure of tissue engineered cartilage constructs. The culture of tissue engineered cartilage constructs was carried out using a cell-polymer composite in two different bioreactor configurations.

The concentric cylinder bioreactor has been used before and validated for tissue engineering of cartilage constructs. In this work, we have identified specific culture conditions that enhance the material properties of the constructs. The use of low oxygen tension to modulate extracellular matrix production is well-documented and this result is reinforced in our studies. Decreasing the shear stress over the culture period leads to increase in construct ECM deposition. The response of chondrocytes to hydrodynamic loading schedules is complex. This is seen in the response of chondrocyte proliferation and matrix deposition to increasing and decreasing hydrodynamic loading. Both low oxygen tension and DHL increase construct GAG by approximately 40%. Combining the two conditions led to an increase in construct collagen content by almost 3-fold compared to the control. The biochemical results were corroborated by histological staining. The shear modulus of the scaffolds cultured in 5% oxygen tension with DHL was in the range of native tissue (200-400 kPa). These results suggest that application of simultaneous stimuli are necessary for increasing construct extracellular matrix deposition.

The response of chondrocytes to hydrodynamic loading in the concentric cylinder bioreactor is complex as shown by these experiments. Multiple biochemical pathways including G-protein signaling and PGE_2 synthesis could be potentially involved in these pathways. In order to resolve and fully understand the effects of varying hydrodynamic loading on chondrocyte metabolism, mechantransduction experiments can be undertaken where the force on individual chondrocytes can be measured using atomic force microscopy to study the effect of different shear stresses.

Preliminary work with TGF-β1 in the presence of DHL showed that the growth factor increased cell proliferation initially, but there was a drop in construct cell number over time. No significant increase in construct ECM was observed in this study. Thus, the effect of TGF-β1 in the presence of DHL does not seem to be synergistic. The effect of TGF-β1 on construct development was not as straightforward as expected. Understanding the mechanism(s) of action of this growth factor are important to harnessing its full potential. Only one concentration of TGF-β1 was studied in this work (0.2 ng/ml). Future studies can use concentrations in a range as wide as 0-50 ng/ml (Blunk et al., 2002). As opposed to having TGF-β1 through the entire culture period, it may be potentially beneficial to use TGF- β1 early in culture to increase chondrocyte proliferation in the construct. TGF- β1 can be removed at later times to minimize any negative effects of the protein on extracellular matrix deposition. Additionally, investigation into the active and latent stages of TGF- β1 would be useful to understand if the effects shown in the studies with exogenous TGF- β1 are

because of the growth factor added to culture or that produced by the chondrocytes inherently. Because TGF- β1 is known to have complex modes of interaction in the presence of nitric oxide, it may be interesting to see if the addition and removal of exogenous TGF- β1 has any effect on construct extracellular matrix deposition.

The concentric cylinder is a very useful tool for manipulating bioreactor conditions for tissue engineering of cartilage. Based on the observations in this study, it would be interesting to investigate the mechanisms and signaling pathways that trigger the production and deposition of cartilaginous matrix in the constructs. The effect of varying shear stress as seen in the hydrodynamic loading studies is complex. The constructs exhibit varying kinetics throughout the culture period under any given hydrodynamic loading condition. Multiple pathways have been postulated to affect the response of chondrocytes to shear stress including gap junctions and calcium signaling pathways. Therefore, it would be necessary to know if the signaling pathways that affect chondrocytes in monolayer culture also affect chondrocytes in three-dimensional culture. For example, it would be interesting to see if intracellular calcium levels change in the chondrocytes, when the shear stress is changed in bioreactor culture. Changes in calcium levels are indicative of intracellular response to extracellular loading signals. Additional pathways that could be investigated include nitric oxide levels in the cells and the levels of inducible nitric oxide synthase levels.

Collagen content increased 3-fold in constructs cultured in hypoxia with DHL as compared to normoxia with DHL. This leads to a 3-4 fold increase in construct dynamic shear modulus, confirming our hypothesis that increase in construct extracellular matrix content leads to an increase in construct material properties. Low oxygen tension is hypothesized to trigger pathways via the hypoxia inducible factor (HIF-1α) (Coimbra et al., 2004; Rajpurohit et al., 1996). It would be interesting to see if this factor is increased in articular chondrocytes in low oxygen tension. Quantitative PCR studies should be carried out to see if mRNA expression of extracellular matrix proteins is in tandem with HIF-1α. Microarray studies may be useful in identifying the genes from the hypoxia responsive elements that are affected by low oxygen and varying shear stress conditions.

Little is known about the ultrastructure of tissue engineered cartilage. It is hypothesized that shear stress applied on the construct surface is likely to promote flattened morphology of chondrocytes while the cells within the construct will continue to be rounded. Fluid flow within and across the construct is hypothesized to affect collagen fibril orientation within the tissue. Organization of matrix within the construct is important for enhanced material properties of the tissue construct. Quantitative morphometric analysis can be carried out by the use of scanning electron microscopy (SEM), transmission electron microscopy (TEM), confocal laser scanning microscopy (CLSM) and stereological methods of analysis. Confocal microscopy should be used to provide visualization and quantification of proliferating cells, matrix organization in higher ultrastructural

detail. CLSM also will allow for 3D reconstruction of the construct by means of optical sectioning. Use of the disector method with CLSM can be used to study the morphology of the cells within the constructs. The disector method allows for optical sectioning of the tissue and reconstruction of the 3-D organization from 2-D images. The spatial orientation of cells in different layers of the construct can be visualized. This information when compared with the cell morphology in native tissue may provide information regarding the spatial organization of engineered cartilage constructs. It is expected that the cell morphology will vary with the bioreactor outer cup rotation rate and the loading regimen. Toluidene blue stained sections of the cartilage should be studied for cell density and quantitative evaluation of cell morphology should be done by mathematical analysis of the intensity functions and cluster densities. This will provide information regarding the cell density within the construct and cellular orientation.

Scanning electron microscopy allows a high spatial resolution, which can be exploited to study the fibril orientation of the collagen fibers and proteoglycans in the construct. The high magnification capabilities of TEM and SEM combined with imaging software (e.g. Image Pro ®) can be used to measure fiber diameters, lengths and periodicity in the constructs. This in turn can be correlated with the bioreactor operating conditions. In order to study the collagen component alone, the proteoglycans can be digested by hyaluronidase and the collagen fiber microstructure studied. Microscopic stereological techniques can be used to measure the collagen volume density, surface density, and length density of the collagen fibers. The matrix fibril orientation is expected to vary with

internal flow within the construct. Samples for stereological analysis can be photographed under transmission electron microscopy. The length of the fibers and their volume and surface densities can be evaluated by means of mathematical calculations. Since the stereological method is based on statistical analysis, it gives a 3D representation of the collagen network in the cartilage construct. These studies will help to obtain correlations between the structure of the construct and its biochemical composition and mechanical properties of the construct. The material properties can be directly related to the fibrillar networking and the organization. Comparing the structure of native cartilage and engineered tissue will allow us to identify the relative merit of engineered constructs. Prior studies have shown that the culture environment has an effect not only on the biochemical composition but also on the fibril orientation. Much emphasis has been laid in the literature on the zonal organization of the tissue, cell shape and cell biosynthetic activity specific to the zone of the tissue [22, 23]. Quantitative morphometric analysis of samples cultured under bioreactor conditions outlined above, and their correlation with (a) mechanical properties, (b) biochemical composition, (c) integrability with native tissue will help identify bioreactor-operating conditions that promote matrix deposition and organization. Thus, the relation between cartilage ultrastructure, biochemical composition, and function can be established.

A perfusion concentric cylinder bioreactor was designed to incorporate axial and radial fluid flow through the construct. The bioreactor prototype was built and seeding studies were carried out. While the seeding efficiency in the

bioreactor after 4 days was only 10%, most of the cells in the construct were viable. The perfusion bioreactor showed robust matrix deposition in two-week construct development studies and the kinetics of chondrocyte proliferation followed previously established models. The preliminary long term experiment (35 days) also showed robust cell proliferation and GAG deposition in the construct. The material properties of the construct after 35 days remained at 10% of the values of native tissue.

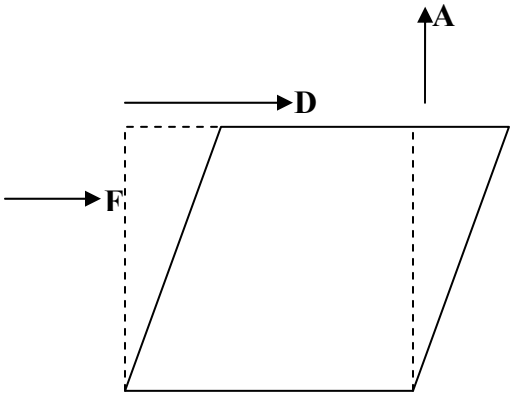
Detailed studies involving different seeding methods should be carried out in the perfusion bioreactor to promote construct seeding efficiency. Some of the experiments that may be carried out here include seeding via the inner tube as opposed to the outer cup and use of a spinner flask to improve seeding efficiency and then transfer of constructs to the perfusion systems. The bioprocessing conditions tested in the concentric cylinder bioreactor such as low oxygen tension and varying hydrodynamic loading can be used in the perfusion bioreactor to enhance construct material properties. The ultrastructural studies outlined above will be useful in evaluating the ability of the perfusion bioreactor in producing constructs with an ultrastructure similar to that of native tissue. Computational fluid dynamic models can be used to understand the complex hydrodynamic environment in the perfusion concentric cylinder bioreactor.

In summary, the current research has identified bioreactor bioprocessing tools for improving construct material properties. Using the information obtained from these conditions, further experiments can be designed using statistical methods to improve the functionality of tissue engineered constructs. Thus, this

research has not only identified important culture conditions for tissue engineering but has also increased the database of available information to allow future researchers to design their experiments using factorial methods.

APPENDIX 1

CALCULATION OF ELASTIC ENERGY STORAGE AND VISCOUS ENERGY DISSIPATION



F is the shear force applied on the tissue construct with cross sectional area A.

Shear Stress $\tau = \frac{F}{A}$

Shear strain $\upsilon = \frac{D}{H}$

Shear rate $\upsilon = \frac{V}{H}$

<u>Oscillation</u>

$\tau^* = \tau e^{-i\delta}$ $\quad \tau^* = \tau' - i\tau''$

Shear stress $\quad \tau'' = \text{elastic stress} = \tau \sin\delta$

$\quad \tau' = \text{viscous stress} = \tau \cos\delta$

Shear strain $\upsilon^* = \upsilon e^{-i\pi/2}$

Shear rate $\upsilon^* = \upsilon e^{-i\omega}$ $\quad \omega = 2\pi f$ $\quad f = \text{frequency}$

$$G^* = G' + iG'' = \frac{\tau^*}{\upsilon^*} = \left(\frac{\tau'}{\upsilon} + i\frac{\tau''}{\upsilon}\right)$$

$$\eta^* = \frac{G^*}{i\omega} \quad , \eta^* = \frac{\tau^*}{\upsilon^*} \qquad \eta' - i\eta'' = \left(\frac{\tau'}{\upsilon} - i\frac{\tau''}{\upsilon}\right)$$

$$J^* = \frac{\upsilon^*}{\tau^*} = \frac{1}{G^*} \qquad J^* = \text{complex compliance}$$

$$F^* = \frac{1}{\eta^*} = \text{complex fluidity}$$

Instantaneous viscous energy loss and elastic energy storage

Energy dissipated per volume per cycle

$$\eta'\upsilon^2\left(\frac{2\pi}{\omega}\right)$$

$$= \tau'\upsilon\left(\frac{2\pi}{\omega}\right)$$

$$= \tau'\upsilon\left(\frac{1}{f}\right) = \frac{G'\upsilon\upsilon'}{f}$$

Average power dissipated per volume = $\eta'\upsilon^2 = \tau'\upsilon$

Maximum elastic energy per volume = $\frac{\eta''\upsilon^2}{\omega} = \tau''\upsilon$

Viscous stress = rate of energy dissipated per unit volume
Elastic stress = maximum energy stored per unit volume

APPENDIX 2

EXPERIMENT DATA - CONSTRUCT BIOCHEMICAL COMPOSITION

Decreasing Hydrodynamic Loading

Cell/Construct (Millions)

76 rpm	12 d	24 d	35d	**DHL**	12d	24d	35d
expt 1	9.002727	6.941688	51.75175		11.58818	7.286688	15.9711
	18.63974	31.09383	84.93974		2.029851	2.637195	39.1461
	4.967159	18.34134	9.95422		19.9122		11.92549
			45.48011		14.99464		5.932753
expt 2	14.6402	10.90525	7.184445		40.5889	8.024798	10.9634
	34.26547	7.206993	7.749422		7.552373	8.016613	38.78762
			8.329706		7.492206		21.81244
							7.954242
expt 3	7.033751	6.938739	9.336272		7.305979	7.826077	7.95316
	8.785054	8.370216	7.912999		7.780863	7.008314	7.79556
			7.78767		7.568608		7.2463
							7.130368
expt 4	8.709093	6.609613	7.329228		7.885474	0.973013	7.148074
	9.15027	9.374226	8.046793		8.446455	0.948828	7.403886
	8.399734		5.996229		7.633217		5.876434
							6.787579
n	10	9	13		13	8	16
Average	12.35932	11.75354	20.13835		11.59838	5.340191	13.11466
SEM	2.730229	2.709912	6.856704		2.696831	1.14005	2.731467

Construct GAG Content(µg)

76 rpm				**DHL**			
	12 d	24 d	35d		12d	24d	35d
expt 1	2117.028	1893.459	3160.35		1669.89	1967.982	9379.798
	1446.321	1669.89	2638.689		1893.459	2638.689	9842.479
	1781.675	1781.675	2899.52		1781.675	2303.336	7487.014
expt 2	343.1439	212.8995	5020.802		3112.579	2577.049	6351.343
	991.8612	325.611	5087.45		4374.435	1288.524	10136.91
	738.8865	4709.779	5184.45		4954.154		4954.154
							4976.37
expt 3	4931.938	4865.29	5702.895		4976.37	5131.882	4954.154
	6610.174	7344.638	6653.378		5242.961	6869.396	6653.378
			6999.008		5242.961		6696.581
							6221.34
expt 4	6437.359	7344.638	6610.174		6178.136	6739.785	7128.619
	6307.748	6523.766	6264.544		5918.914	6782.989	6869.396
	8035.898		5659.691		6566.97		8035.898
							7690.268

n	12	10	12	12	9	15
average	3612.912	3667.164	5156.746	4326.042	4033.292	7158.514
SEM	824.7847	891.4313	434.9335	510.5659	772.7466	429.4631

Construct Collagen Content(µg)

76 rpm	12 d	24 d	35d	12d	24d	35d
expt 1	163.8468	2075.393	764.6184	273.078	436.9248	655.3872
	436.9248	764.6184	436.9248	710.0028	928.4652	600.7716
	2621.549		5352.329	983.0808	5516.176	3058.474
				2621.549	1693.084	
expt 2	3604.63	2293.855	7952.031	600.7716	764.6184	5898.485
	2785.396	1474.621	3550.014	2403.086	2949.242	5734.638
		4423.864		1310.774	2184.624	1201.543
expt 3	4205.401	928.4652	4696.942	7646.184	2184.624	710.0028
	542.8791	196.6162	7799.108	6390.025	1365.39	240.3086
			3593.706	3702.938	7919.262	142.0006
expt 4	5439.714	2173.701	3866.784	2020.777	3189.551	3582.783
	8956.958	4631.403	2479.548	3047.55	316.7705	775.5415
			382.3092	1245.236	415.0786	218.4624

n	9	9	11	13	13	12
average	3195.255	2106.948	3715.847	2535.004	2297.216	1901.533
SEM	940.9868	513.9881	884.5971	749.4464	742.8471	710.2726

Increasing Hydrodynamic Loading

Construct Cell Number (Millions)

19 rpm	12 day	24 day	35 day	**IHL**	12 day	24 day	35 day
expt 1	4.041732	6.98837	10.08198		2.748554	7.272318	11.80115
	1.952315	13.07617	18.00671		7.554297	8.772629	9.397871
	1.97338	14.99442	10.46235		3.840386	10.85885	10.63656
							7.452111
expt 2	6.41647	12.05533	12.47236		7.575284	4.11067	11.30382
	1.109278	11.10958	6.359932		8.869518	5.574708	11.75474
	2.922893	16.02163	13.9335		8.429943	6.742922	8.961646
	1.867555						10.36334
							10.45478
expt 3	6.879032	3.239386	27.39406		7.488024	5.524953	7.970855
	7.737782	7.2843	26.93971		6.868618	8.069619	8.277957
	0.76026	15.67181	8.588147		5.426296	7.196224	19.27514
	2.46501				2.599178		6.068605
							11.77562
expt 4	1.58841	6.659525	24.739		5.350973	1.239091	16.38967
	3.662963	3.009476	17.05181		5.065799	5.162855	2.854039
	0.083766	3.128453	17.34055		6.025555	4.795839	15.40025
					3.490597		16.79887
							14.27631
n	**14**	**12**	**12**		**14**	**12**	**19**
Average	**3.104346**	**9.436538**	**16.11418**		**5.809502**	**6.276723**	**11.11649**
SEM	**0.634851**	**1.440427**	**1.984054**		**0.555784**	**0.712737**	**0.921323**

Construct GAG Content (µg)

19 rpm	12 day	24 day	35 day	**IHL**	12 day	24 day	35 day
expt 1	3134.294	3585.461	6170.615		567.252	1364.204	10186.66
	1509.104	4446.63	6341.861		4830.286	1461.353	5108.561
	2299.47	6160.735	3089.836		1010.186	5385.189	5811.657
							3295.66
expt 2	1138.62	4517.433	4520.727		801.0685	3723.775	4143.656
	1372.437	4398.879	2828.027		5887.4	1108.982	5770.492
	1069.463	7048.25	9521.436		2304.41	2271.478	4879.684
	1709.989						2072.24
							4150.243
expt 3	3876.908	1922.4	5483.985		1919.106	1336.212	2711.119
	3353.291	3918.073	13678.26		1639.185	3559.115	4456.509
	1326.332	2250.072	23409.64		2353.808	876.8118	4664.804
	2516.82				1691.876		3350.821
							7549.638
Expt 4	4530.606	5307.799	8784.584		4716.671	806.0083	3110.418
	412.472	1438.301	6262.001		5487.278	1104.042	8333.417
	3796.225	1882.881	4197.171		4631.048	2338.988	8712.134
					1357.618		7895.423
n	**14**	**12**	**12**		**14**	**12**	**19**
Average	**2289.002**	**3906.409**	**7857.346**		**2799.8**	**2111.346**	**5344.619**
SEM	**337.7327**	**513.4101**	**1598.204**		**502.5366**	**411.2103**	**529.8728**

Construct Collagen Content (µg)

19 rpm

	12 day	24 day	35 day	**IHL**	12 day	24 day	35 day
expt 1	1170.294	528.7734	923.9785		1027.69	1105.867	970.7274
	1062.261	1899.813	1142.795		194.8526	1041.833	2655.26
	816.731	1091.332	1798.458		748.7683	1279.113	964.8347
			2362.195				7160.048
							985.2628
expt 2	1414.646	1098.01	544.0945		1822.815	1089.368	1802.387
	1447.252	1354.54	1466.894		2172.842	912.1931	1055.975
	2134.343	1546.643	867.8012		955.4063	1343.147	1277.542
	1280.292						4160.653
							1380.861
expt 3	1960.312	1213.9	1346.683		1248.078	1908.063	2570.404
	879.5867	872.1226	2016.882		1853.85	1239.043	1601.248
	875.2653	1172.651	1804.744		428.2043	1122.367	914.5501
	924.7642				1097.617		1566.678
							1243.757
expt 4	1446.466	698.4837	1455.109		1029.262	1625.605	1360.825
	540.5588	423.883	2108.022		1048.511	1247.685	2736.579
	621.0927	977.7987	2724.008		1088.582	1469.252	2027.881
					1077.975		1437.038
n	**14**	**12**	**12**		**14**	**12**	**19**
Average	**1183.847**	**1073.163**	**1581.666**		**1128.175**	**1281.961**	**1993.29**
SEM	**124.9711**	**120.7671**	**174.9198**		**142.2143**	**79.76777**	**343.742**

Low oxygen tension with decreasing hydrodynamic loading

Construct Cell Number (Millions)

20% oxygen + DHL	12 day	24 day	35 day	5% oxygen + DHL	12 day	24 day	35 day
expt 1	64.48086	53.36177	43.96944		1.519816	4.835764	12.97759
	55.02732	38.00494	60.3043		2.599052	2.59235	27.28881
	67.73112	35.79288	57.24447		2.224082	42.44328	19.10566
	25.15386	30.07108	63.33123		1.814682	29.65581	72.12816
			53.09127				104.0206
			60.90484				
expt 2	4.898804	17.7322	22.09007		27.35551	19.45642	92.53381
	8.161651	18.50693	19.91592		40.77179	13.14354	101.4078
	15.51148	28.30995	7.95647		36.12598	14.06828	92.35054
	2.993293	16.74764	2.800421		29.35031	13.03769	108.5773
		23.42123	12.85186				5.054831
			21.57774				
expt 3	1.003678	28.18375	1.475205		1.175277	0.427827	0.711105
	1.305476	24.98347	0.456972		0.580195	0.481056	0.437724
	16.94632	18.91651	0.408311		0.422754	0.537113	1.57308
	18.55625	17.95076	0.934458		0.422338	1.907174	19.01722
		20.98409	1.837804				19.52914
			1.604889				
expt 4					46.52157	27.07726	21.18895
					34.28808	37.83671	27.16644
					23.95476	37.68096	28.98539
					21.29424	39.36394	4.567787
						30.14248	0.64466
						23.4334	
n	**12**	**14**	**18**		**16**	**18**	**20**
Average	**23.48084**	**26.64051**	**24.04198**		**16.90128**	**18.7845**	**37.96333**
SEM	**7.158492**	**2.960789**	**5.879337**		**4.033803**	**3.558555**	**8.953166**

Construct GAG Content (µg)

20% oxygen + DHL	12 day	24 day	35 day	5% oxygen + DHL	12 day	24 day	35 day
expt1	11978.2	18814.31	35887.91		2007.482	2881.17	2714.436
	5215.451	28851.72	23056.03		2621.064	2841.154	3834.891
	19834.72	34874.16	30865.87		2747.783	35027.56	3894.915
	3521.43	52554.68	27604.54		2761.121	3721.511	26497.43
		1949.578	33933.78				39669.44
			34714.1				
expt 2	1023.046	1660.037	1666.471		20975.18	31699.54	34980.87
	1164.599	2934.018	913.6635		39362.65	29765.42	36194.7
	2882.544	2766.728	1434.838		37848.7	34167.21	35527.76
	1614.997	3428.059	3313.639		31045.94	32553.22	36181.36
			1927.449				1415.535
			2354.936				

expt 3	1472.205	3641.479	1687.352		302.4097	1376.93	1241.81
	960.39	2961.203	1900.772		1524.917	1177.468	894.3607
	2861.162	660.2681	1827.409		1106.691	1595.694	18474.17
	7543.063	1740.707	1920.78		1396.232	1666.471	20101.5
		4188.368	820.3331				20368.27
			2670.214				
expt 4					19494.58	20721.75	13885.64
					18881	19567.95	15606.34
					18934.36	18480.84	1415.535
					13132	13038.63	1621.431
						17333.71	598.3852
						20381.61	
n	**12**	**14**	**18**		**16**	**18**	**20**
Average	**5005.984**	**11501.81**	**12107.64**		**13383.88**	**15999.88**	**15755.94**
SEM	**1645.173**	**4315.91**	**3393.035**		**3231.13**	**2877.647**	**3268.268**

Construct Collagen Content (µg)

20% oxygen + DHL				**5% oxygen + DHL**		
expt 1	12 day	24 day	35 day	12 day	24 day	35 day
	1693.084	5002.789	5363.252	994.0039	17902.99	9918.193
	6848.796	4150.786	6859.719	6237.102	9743.423	2250.163
	12528.82	3080.32	6127.87	10103.89	10868.5	17739.15
	3451.706	1776.045	199.9188	1769.545	17804.69	15805.75
		780.1596	199.9188		17673.61	13948.82
			599.7564			
expt 2	3598.538	393.2323	2548.965	7471.414	15095.75	17444.22
	199.9188	399.8376	999.594	13074.97	17870.22	12845.59
	299.8782	299.8782	299.8782	16351.91	17892.07	13479.13
	5347.828	999.594	199.9188		349.8579	6717.719
			1728.763			199.9188
expt 3	789.0251	199.9188	2240.004	17859.3	349.8579	149.9391
	292.5599	1832.193	854.0384	449.8173	249.8985	199.9188
	1028.392	2242.959	2724.648	199.9188	2417.313	149.9391
	1554.409	2157.26	1575.095	749.6955	249.8985	4198.295
		1190.925	1270.715			149.9391
expt 4				1424.382	2627.128	3723.489
				1675.57	3891.933	2739.424
				1604.646	3286.127	2086.336
				6049.192	3368.871	3593.462
					2325.703	3094.042
n	**12**	**14**	**18**	**16**	**18**	**20**
Average	**3136.079**	**1750.421**	**2112.003**	**5734.357**	**7998.214**	**6521.672**
SEM	**1053.489**	**382.4086**	**541.3063**	**1558.069**	**1657.98**	**1427.147**

Perfusion Bioreactor

14 day construct Biochemical Composition

Construct Cell Number (Millions)

	4day	7day	11day	14 day
Expt 1	0.069215	0.106544	1.691224	4.033348
	1.248966	0.367764	2.042982	3.357293
	0.332375	0.132414	1.729227	7.053816
	2.004355	2.292804		4.150439
Expt 2	1.999792	0.613806	3.148085	3.038532
	0.844897	0.123081	1.771753	2.291915
	0.560352	1.330519	3.902716	3.05397
	1.382626	0.86415		1.751866
Expt 3	0.574147	0.51832	0.661408	1.861771
	1.822058	0.571758	1.467725	1.947975
	1.799932	0.95991	1.771423	1.944853
Expt 4	1.003232	0.259024	3.553695	3.605172
	0.242039	2.286093	1.189228	5.381606
	2.648649	3.06833	0.583269	2.362878
		1.002954		
Average	**1.180903**	**0.966498**	**1.959395**	**3.27396**
SEM	**0.210671**	**0.234512**	**0.305841**	**0.405492**

Construct GAG Composition (µg)

	4day	7day	11day	14day
Expt 1	2607.726	2714.436	2581.048	4755.264
	3161.284	3134.606	3394.712	4948.676
	3074.582	3194.631	2707.766	8756.889
	3035.866	1694.021		9297.109
Expt 2	2127.253	2899.932	1571.591	1948.392
	1805.303	1390.346	1767.146	1390.346
	2024.706	2062.863	2065.248	2062.863
	4171.037	1793.379		
Expt 3	1545.359	686.826	2353.81	15625.29
	2961.937	1201.946	2081.941	23566.72
	2275.111	1108.938	1874.463	20018.12
				833.1133
Expt 4	1343.183	25407.12	1445.196	28886.93
	6795.258	3383.46	4925.003	969.1318
	6103.83	4006.878	3087.921	21749.49
Average	**3073.745**	**3905.67**	**2487.987**	**10343.45**
SEM	**432.5226**	**1618.316**	**280.1905**	**2601.543**

Construct Collagen Composition (µg)

	4day	7day	11day	14day
Expt 1	620.3696	510.5837	596.6783	1121.428
	302.4912	779.492	1138.647	924.082
	542.3349	311.406	1014.084	1023.427
	612.3707	896.6661		1243.792
Expt 2	779.6141	1276.581	1376.598	1402.976
	1460.311	248.0257	1358.829	1317.797
	1082.777	5762.66	1966.071	2035.13
	1435.948	638.9318		1024.831
Expt 3	886	736.6278	1341.794	1272.246
	1513.555	1207.767	347.1261	607.547
	862.0451	782.9113	810.2051	823.5773
		1224.192		
Expt 4	729.6059	1836.929	2243.222	775.7673
	1374.949	1441.627	198.8114	1995.197
	377.9614	7818.252	2748.066	1600.993
Average	**898.5952**	**1698.177**	**1261.678**	**1226.342**
SEM	**109.9487**	**554.2496**	**219.962**	**113.8706**

35 day constructs

Construct Cell Number (millions)

	4 day	12 day	20 day	28 day	35 day
	0.22786	5.811075	7.432704	3.369997	3.709642
	0.927246	7.70632	7.305018	6.251951	1.862018
	1.291038	5.401631	6.971898	9.364972	6.565469
Average	**0.815382**	**6.306342**	**7.23654**	**6.328973**	**4.213743**
SEM	**0.311968**	**0.709898**	**0.137359**	**1.731029**	**1.920176**

Construct GAG composition (µg)

	4 day	12 day	20 day	28 day	35 day
	4294.827	6031.048	5681.822	5288.015	4914.021
	3947.252	5283.061	5041.988	4774.496	3921.659
	5117.943	6725.371	5794.103	6081.409	12150.34
					5366.446
Average	**4453.341**	**6013.16**	**5505.971**	**5381.307**	**6588.116**
SEM	**347.1185**	**416.4551**	**234.2448**	**380.146**	**1878.461**

Construct Collagen Composition (µg)

	4 day	12 day	20 day	28 day	35 day
	744.3329	449.1628	616.164	378.2322	750.8424
	983.1626	1007.18	417.0644	599.7781	917.1702
	813.6923	467.1199	283.0594	1899.874	1090.456
					1414.358
Average	**847.0626**	**641.1543**	**438.7626**	**959.2946**	**1043.207**
SEM	**70.93445**	**183.0864**	**96.76909**	**474.6182**	**141.8179**

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